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NOVEL INHIBITOR OF NF-κB ACTIVATION

Field of the Invention

The present invention is concerned with a novel protein that inhibits the activation of transcription factor NF-κB by various signals that are important in inflammatory and immune processes. More particularly the invention relates to a protein herein designated NAP (Nemo Associated Protein), its recombinant production and its use.

This application is one for a patent of addition to Israel Patent Application No. 126024. The main patent, among others, discloses a clone-10, but its full sequence was not yet elucidated.

Background of the Invention

The Tumor Necrosis Factor/Nerve Growth Factor (TNF/NGF) receptor superfamily is defined by structural homology between the extracellular domains of its members [Bazan, J. F. (1993). Current Biology 3, 603-606]. In general, with the exception of two receptors, the p55 TNF receptor and Fas/APO1, the various members of this receptor family do not exhibit clear similarity of structure in their intracellular domains. Nevertheless, there is much similarity of function between the receptors, indicating that they share common signaling pathways. This is seen in the ability of several receptors of the TNF/NGF family to activate the transcription factor NF-κB, by means of a cytoplasmic activator protein, TNF Receptor Associated Factor 2 (TRAF2). TRAF2 exerts its activity by binding to the structurally-dissimilar intracellular domains of several of the receptors of the TNF/NGF family.

TRAF2 is a member of a recently described family of proteins called TRAF (TNF Receptor Associated Factor) that includes several proteins identified as, for example, TRAF1, TRAF2, TRAF3, and TRAF6.

All proteins belonging to the TRAF family share a high degree of amino acid identity in their C-terminal domains, while their N-terminal domains may be unrelated. The TRAF2 molecule contains a ring finger motif and two TFIIIA-like zinc finger motifs at its C-terminal area. The C-terminal half of the molecule includes a region known as the "TRAF domain" containing a potential leucine zipper region extending between amino acids 264 - 358 (called N-TRAF), and another part towards the carboxy end of the domain between amino acids 359 - 501 (called C-TRAF) which is responsible for TRAF binding to the receptors and to other TRAF molecules to form homo- or heterodimers.

Activation of the transcription factor NF-κB is one manifestation of the signaling cascade initiated by some of the TNF/NGF receptors and mediated by TRAF2. NF-κB comprises members of a family of dimer-forming proteins with homology to the Rel oncogene which, in their dimeric form, act as transcription factors. These factors are ubiquitous and participate in regulation of the expression of multiple genes. Although initially identified as a factor that is constitutively present in B cells at the stage of Igκ light chain expression, NF-κB is known primarily for its action as an inducible transcriptional activator. NF-κB has many different activities in the cell, most of which are rapidly induced in response to extracellular stimuli. The majority of the NF-κB-activating agents are inducers of immune defense, including components of viruses and bacteria, cytokines that regulate immune response, UV light and others. Accordingly,

many of the genes regulated by NF-κB contribute to immune defense [Grilli, M., Chiu, J. J., and Lenardo, M. J. (1993). Int RevCytol.].

One major feature of NF-κB-regulation is that this factor can exist in a cytoplasmic non-DNA binding form which can be induced to translocate to the nucleus, bind DNA and activate transcription. The switching between these two forms of NF-κB is regulated by I-κB - a family of proteins that contain repeats of a domain that was initially identified in the erythrocyte protein ankyrin [Gilmore, T. D., and Morin, P. J. (1993) Trends Genet 9, 427-33.] In the unstimulated form, the NF-κB dimer occurs in association with an I-κB molecule which causes retention of the dimer in the cytoplasm and prevents its interaction with the NF-κB-binding DNA sequence and subsequent activation of transcription. The dissociation of I-κB from the NF-κB dimer constitutes the critical step of its activation by many of its inducing agents [DiDonato, J. A., Mercurio, F., and Karin, M. (1995). Mol Cell Biol 15, 1302-11].

One of the most potent inducing agents of NF-κB is the cytokine tumor necrosis factor (TNF). There are two different TNF receptors, the p55 and p75 receptors, the expression levels of which differ according to cell type [Vandenabeele, P., Declercq, W., Beyaert, R., and Fiers, W. (1995) Trends Cell Biol. 5, 392-400]. The p75 receptor responds preferentially to the cell-bound form of TNF (TNF is expressed both as a beta-transmembrane protein and as a soluble protein) while the p55 receptor responds equally to both forms of TNF [Grell, M. et al., (1995) Cell 83, 793-802]. The intracellular domains of the two receptors are structurally unrelated and bind different cytoplasmic proteins. Nevertheless, at least some of the effects of TNF,

including the cytocidal effect of TNF and the induction of NF-κB, can be induced by both receptors. This feature is cell specific. The p55 receptor is capable of inducing a cytocidal effect or activation of NF-κB in all cells that exhibit such effects in response to TNF. The p75-R can have such effects only in some cells. Others, although expressing the p75-R at high levels, show induction of the effects only in response to stimulation of the p55-R [Vandenabeele, P., Declercq, W., Beyaert, R., and Fiers, W. (1995). Trends Cell Biol. 5, 392-400]. Apart from the TNF receptors, various other receptors of the TNF/NGF receptor family: CD30 [McDonald, P. P. et al., (1995). Eur J Immunol 25, 2870-6, CD40 [Berberich, I., Shu, G. L., and Clark, E. A. (1994).. J Immunol 153, 4357-66]., the lymphotoxin beta receptor and, in a few types of cells, Fas/APO1 [Rensing-Ehl, A. *et al.* (1995). J. Inlamm. 45, 161-174, are also capable of inducing activation of NF-κB. The IL-1 type I receptor, which also effectively triggers NF-κB activation, shares most of the effects of the TNF receptors despite the fact that it has no structural similarity to them.

The activation of NF-κB upon triggering of these various receptors results from induced phosphorylation of its associated I-κB molecules. This phosphorylation tags I-κB for degradation, which most likely occurs in the proteasome. The nature of the kinase that phosphorylates I-κB, and its mechanism of activation upon receptor triggering is still unknown. However, in recent years, there have been some advantages in relation to the identity of three receptor-associated proteins that appear to take part in initiation of the above-mentioned phosphorylation. A protein called TRAF2, initially cloned by D. Goeddel and his colleagues [Rothe, M. *et al.* (1994). Cell 78, 681-692 , seems to play a central role in NF-κB-activation by the various receptors of the TNF/NGF

family. The protein, which when expressed at high levels can by itself trigger NF-κB activation, binds to activated p75 TNF-R, lymphotoxin beta receptor [Mosialos, G. *et al.* (1995) Cell 80, 389-399], CD40 [Rothe, M. *et al.* (1995) Science 269, 1424-1427 and CD-30 (unpublished data) and mediates the induction of NF-κB by them. TRAF2 does not bind to the p55 TNF receptor nor to Fas/APO1, however, it can bind to a p55 receptor-associated protein called TRADD and TRADD has the ability to bind to a Fas/APO1-associated protein called MORT1 (or FADD). Another receptor-interacting protein, called RIP [Stanger, B.Z. et al. (1995) Cell 81, 513-523 is also capable of interacting with TRAF2 as well as with FAS/APO1, TRADD, the p55 TNF receptor and MORT-1. Thus, while RIP has been associated with cell cytotoxicity induction (cell death), its ability to interact with TRAF2 also implicates it in NF-κB activation and it also may serve in addition to augment the interaction between FAS/APO1, MORT-1, p55 TNF receptor and TRADD with TRAF2 in the pathway leading to NF-κB activation. These associations apparently allow the p55 TNF receptor and Fas/APO1 to trigger NF-κB activation [Hsu, H., Xiong, J., and Goeddel, D. V. (1995) Cell 81, 495-504.]. The triggering of NF-κB activation by the IL-1 receptor occurs independently of TRAF2 and may involve a recently-cloned IL-1 receptor-associated protein-kinase called IRAK [Croston, G. E., Cao, Z., and Goeddel, D. V. (1995) J Biol Chem 270, 16514-7.]

By what mechanism TRAF2 acts is not clear. Several cytoplasmic molecules that bind to TRAF2 have been identified. However, the mechanisms by which TRAF2, which by itself does not possess any enzymatic activity, triggers the phosphorylation of I-κB is still uncertain.

A protein called RAP-2 (RIP associated protein-2), now known as NEMO, is disclosed in WO 99/47672, which also discloses clone 10 herein.

In addition, it is to be noted that TRAF2 also binds to the p55 (CD120a) and p75 (CD120b) TNF receptors, as well as to several other receptors of the TNF/NGF receptor family, either directly or indirectly via other adpator proteins as noted above. TRAF2 is thus crucial for the activation of NF-κB [Wallach, D. (1996) Eur. Cytokine Net. 7, 713-724.] However, TRAF3 actually inhibits activation of NF-κB by some receptors of the TNF/NGF family, whilst TRAF6 is required for induction of NF-κB by IL-1 [Cao, Z. *et al.* (1996) Nature 383, 443-446.]

It is now known (see brief reviews and references in, for example, co-pending co-owned Israel Patent Application Nos. 114615, 114986, 115319, 116588), that TNF and the FAS/APO1 ligand, for example, can have both beneficial and deleterious effects on cells. TNF, for example, contributes to the defence of the organism against tumors and infectious agents and contributes to recovery from injury by inducing the killing of tumor cells and virus-infected cells, augmenting the antibacterial activities of granulocytes, and thus in these cases the TNF-induced cell killing is desirable. However, excess TNF can be deleterious and as such may play a major pathogenic role in a number of pathological states such as septic shock, anorexia, rheumatic diseases, inflammation and graft-vs-host reactions. In such cases the TNF-induced cell killing has a deleterious effect. The FAS/APO1 ligand, for example, also has both desirable and deleterious effects. Binding of this ligand to its receptor induces the killing of autoreactive T cells during maturation of T cells, i.e. the killing of T cells which recognize self-antigens, during their development, thereby preventing the occurrence of autoimmune diseases. Further, various malignant cells and HIV-infected

cells carry the FAS/APO1 receptor on their surface and can thus be destroyed by activation of this receptor by its ligand or by antibodies specific thereto, and thereby activation of cell death (apoptosis) intracellular pathways mediated by this receptor. However, the FAS/APO1 receptor may mediate deleterious effects, for example, uncontrolled killing of tissue which is observed in certain diseases such as acute hepatitis that is accompanied by the destruction of liver cells.

NF-κB is known to control the expression of many immune- and inflammatory-response genes. Thus, in view of the fact that the TNF/NGF family of receptors can induce cell survival pathways (via NF-κB induction) on the one hand and can induce cell death pathways on the other hand, there apparently exists a fine balance, intracellularly between these two opposing pathways. For example, when it is desired to achieve maximal destruction of cancer cells or other infected or diseased cells, it would be desired to have TNF and/or the FAS/APO1 ligand inducing only the cell death pathway without inducing NF-κB. Conversely, when it is desired to protect cells such as in, for example, inflammation, graft-vs-host reactions, acute hepatitis, it would be desirable to block the cell killing induction of TNF and/or FAS/APO1 ligand and enhance, instead, their induction of NF-κB, which would in turn lead to the enhanced expression of many immune- and inflammatory-response genes. Likewise, in certain pathological circumstances it would be desirable to block the intracellular signaling pathways mediated by the p75 TNF receptor and the IL-1 receptor, while in others it would be desirable to enhance these intracellular pathways.

It is an object of this invention to provide clones, proteins, and other tools for the modulation and/or mediation

of NF-κB effects, in particular clones comprising the NAP protein and the NAP protein itself.

Other objects and advantages of the invention will become apparent as the description proceeds.

SUMMARY OF THE INVENTION

It has now been surprisingly found, and this is an object of the invention, that a protein having the sequence shown in fig. 3 is able to specifically bind to certain key proteins that are implicated in the activation of NF-κB. Furthermore, following binding of the protein of the invention to TNF pathway signaling molecules, the activation of NF-κB is inhibited.

It is thus an object of the invention to provide a novel protein, NAP, including all isoforms, analogs, fragments or derivatives thereof which are capable of binding to the tumor necrosis factor receptor-associated 2 (TRAF2) protein and to one or more proteins of the signalosome (NF-κB complex). As TRAF2 is involved in the modulation or mediation of the activation of the transcription factor NF-κB, which is initiated by some of the TNF/NGF receptors, as well as others as noted above, the novel protein of the present invention by binding to TRAF2 and to signalosome proteins is therefore capable of affecting (modulating or mediating) the intracellular signaling processes initiated by various ligands (e.g. TNF, FAS ligand and others) binding to their receptors such as, for example, their modulation/mediation of NF-κB activation, via interaction directly or indirectly with TRAF proteins and/or with the signalosome or signalosome-interacting proteins.

The novel protein of the present invention is therefore a direct modulator/mediator of the intracellular

biological activity of TRAF2 and signalosome (e.g. induction of NF-κB activation by TRAF2).

The novel protein of the invention is likewise an indirect modulator/mediator of the intracellular biological activity of a variety of other proteins which are capable of interacting with TRAF2 directly (e.g. P75 TNF receptor) or indirectly (e.g. p55 TNF receptor) by means of their associated proteins, such as, for example, TRADD and RIP).

Another object of the invention is to provide antagonists (e.g. antibodies, peptides, organic compounds, or even some isoforms) to the above novel TRAF2-binding protein, including isoforms, analogs, fragments and derivatives thereof, which may be used to inhibit the signaling process, or, more specifically, to inhibit the activation of NF-κB and its associated involvement in cell-survival processes, when desired. Likewise, when the TRAF2-binding protein of the invention is itself inhibitory for NF-κB activation, then it is an object to provide antagonists to this TRAF2-binding protein to activate the signaling process or more specifically, to block the inhibition of NF-κB activation and hence bring about enhanced NF-κB activation, when desired.

A further object of the invention is to use the above novel TRAF2-binding protein, isoforms, analogs, fragments and derivatives thereof, to isolate and characterize additional proteins or factors, which may be involved in regulation of TRAF2/ signalosome activity e.g. other proteins which may bind to TRAF2 proteins and to signalosome proteins and influence their activity, and/or to isolate and identify other receptors or other cellular proteins further upstream or downstream in the signaling process(es) to which this novel protein, analogs, fragments and derivatives bind, and hence, in whose function they are also involved.

A still further object of the invention is to provide inhibitors which can be introduced into cells to bind or interact with the novel protein of the invention and possible isoforms thereof, which inhibitors may act to inhibit NF-κB activation, e.g., TRAF2- mediated NF-κB activation; or which may directly activate the signalosome complex, and hence, when desired, to enhance NF-κB activation.

Moreover, it is an object of the present invention to use the above-mentioned novel protein, isoforms and analogs, fragments and derivatives thereof as antigens for the preparation of polyclonal and/or monoclonal antibodies thereto. The antibodies, in turn, may be used, for example, for the purification of the new proteins from different sources, such as cell extracts or transformed cell lines.

Furthermore, these antibodies may be used for diagnostic purposes, e.g. for identifying disorders related to abnormal functioning of cellular effects mediated directly by TRAF proteins, the signalosome, or by the p55 TNF receptor, FAS/APO1 receptor, or other NF-κB inducing receptors and their associated cellular proteins (e.g. MORT-1, TRADD, RIP), which act directly or indirectly to modulate/mediate NF-κB induction, and other cellular processes, e.g., via interaction with TRAF2.

A further object of the invention is to provide pharmaceutical compositions comprising the above novel protein, isoforms, or analogs, fragments or derivatives thereof, as well as pharmaceutical compositions comprising the above noted antibodies or other antagonists.

In accordance with the present invention, a novel protein, in particular, a TRAF2- and NEMO-binding protein, has been isolated. This protein has high specificity of binding to TRAF2 and to NEMO (see Examples below) and hence is a modulator or mediator of TRAF2/NEMO intracellular activity.

TRAF2 is involved in the modulation or mediation of at least one intracellular signaling pathway being the cell survival- or viability- related pathway in which TRAF-2 is directly involved in activation of NF-κB which plays a central role in cell survival. Further, TRAF2 by being capable of interacting directly or indirectly with the above noted p55 TNF receptor, p75 TNF receptor, FAS/APO1 receptors and their associated proteins MORT-1, TRADD and RIP, also is a mediator or modulator of the NF-κB induction or activation activity attributed to these receptors. TRAF2 is therefore a modulator/mediator of the cell survival pathways (as opposed to the cell death pathways) mediated by these receptors and their associated proteins and as such the extent of interaction between these receptors and/or proteins with TRAF2 and of TRAF-2 with downstream proteins leading to NF-κB induction is an important factor in the outcome of the activity of these receptors. Accordingly, NAP, plays a key role in this interaction between TRAF-2 proteins and components of the signalosome, by binding specifically to TRAF-2 and to components of the signalosome, e.g., to NEMO, and thereby modulate the activity thereof and/or have their activity modulated by interaction with TRAF2 and/or components of the signalosome.

The TRAF2/signalosome interacting protein, NAP, has been isolated and cloned using the two-hybrid system, partially and fully sequenced, and characterized, and as is detailed herein below appears to be a highly specific binding protein, and hence a specific TRAF2/NEMO modulator/mediator.

As will be used herein throughout, TRAF2 activity, is meant to include its activity in modulation/mediation of NF-κB induction/activation. Likewise, as used herein throughout, TRAF2-binding protein activity is meant to include the modulation/mediation of TRAF2- activity by virtue of

specific binding to TRAF2 proteins, this modulation/mediation including modulation/mediation of cell survival pathways, in particular, those relating to NF-κB activation/induction in which TRAF2 is involved directly or indirectly and as such TRAF2-binding protein may be considered as indirect modulator/mediators of all the above mentioned proteins and possibly a number of others which are involved in cell survival, especially NF-κB activation/induction and to which TRAF2 binds, or with which TRAF2 interacts in a direct or indirect fashion.

Accordingly, the present invention provides a DNA sequence encoding NAP, or an isoform, fragment or derivative of a protein capable of binding to TRAF2 and independently to a component of the signalosome.

NAP is capable of binding to TRAF2 and to NEMO.

NAP is a protein capable of binding to at least amino acids 218 to 416 of NEMO.

Other embodiments of the DNA sequence of the invention include :

(a) a cDNA sequence of the herein designated clone 10 comprising the nucleotide sequence depicted in Fig 1;

(b) a cDNA sequence of the herein designated clone compl. 10 comprising the nucleotide sequence depicted in Fig. 2;

(c) a fragment of a sequence (a) or (b) which encodes a biologically active protein capable of binding to at least amino acids 218 to 416 of NEMO;

(d) a DNA sequence capable of hybridization to a sequence of (a)-(c) under moderately stringent conditions and which encodes a biologically active protein capable of binding to at least amino acids 218 to 416 of NEMO; and

(e) a DNA sequence which is degenerate as a result of the genetic code to the DNA sequences defined in (a)-(d) and which encodes a biologically active protein capable of binding to at least amino acids 218 to 416 of NEMO.

Yet other embodiments of the DNA sequence of the invention noted above include a DNA sequence which is either the sequence contained in the herein designated cDNA clone known as clone 10, or the sequence contained in herein designated clone compl. 10, the latter encoding NAP.

Embodiments of the above DNA sequence of the invention encoding the protein NAP include :

(i) A DNA sequence encoding the protein NAP, isoforms, fragments or analogs thereof, said NAP, isoforms, fragments or analogs thereof being capable of binding to TRAF2 and to NEMO and which is capable of modulating the activity of NF-κB;

(ii) A DNA sequence as in (i) above, selected from the group consisting of :

a) a cDNA sequence derived from the coding region of a native NAP protein;

b) DNA sequences capable of hybridization to a sequence of (a) under moderately stringent conditions and which encode a biologically active NAP; and

c) DNA sequences which are degenerate as a result of the genetic code to the sequences defined in (a) and (b) and which encode a biologically active NAP protein;

(iii) A DNA sequence as in (i) or (ii) above comprising at least part of the sequence depicted in Fig. 2 and encoding at least one active NAP protein, isoform, analog or fragment;

(iv) A DNA sequence as in (iii) above encoding a NAP protein, isoform, analog or fragment having at least part of the amino acid sequence depicted in Fig. 3.

In another aspect, the invention provides proteins or polypeptides encoded by the above noted DNA coding sequences of the invention, the isoforms, analogs, fragments and derivatives of said proteins and polypeptides, provided that they are capable of binding to TRAF2 and/or to NEMO, preferably to at least the 222-501 amino acid sequence of TRAF2 and/or to the amino acid sequence of NEMO.

In yet another aspect, the invention provides a vector comprising any of the above DNA sequences according to the invention which are capable of being expressed in host cells selected from prokaryotic and eukaryotic cells; and the transformed prokaryotic and eukaryotic cells containing said vector.

The invention also provides a method for producing a protein, isoform, analog, fragment or derivative encoded by any of the above DNA sequences according to the invention which comprises growing the above mentioned transformed host cells under conditions suitable for the expression of said protein, isoforms, analogs, fragments or derivatives, effecting post-translational modification, as necessary, for obtaining said protein, isoform, analogs, fragments or derivatives and isolating said expressed protein, isoforms, analogs, fragments or derivatives.

In a further aspect, the invention provides antibodies or active fragments or derivatives thereof, specific for the above proteins of the invention, analogs, isoforms, fragments or derivatives thereof or specific for the NAP protein, isoform, analog, fragment or derivative thereof noted above.

In a different aspect, the invention provides the following screening methods:

(i) A method for screening of a ligand capable of binding to a protein according to the invention, as noted above, including isoforms, analogs, fragments or derivatives thereof, comprising contacting an affinity chromatography matrix to which said protein, isoform, analog, fragment or derivative is attached with a cell extract whereby the ligand is bound to said matrix, and eluting, isolating and analyzing said ligand.

(ii) A method for screening of a DNA sequence coding for a ligand capable of binding to a protein, isoform, analog, fragment or derivative according to the invention as noted above, comprising applying the yeast two-hybrid procedure in which a sequence encoding said protein, isoform analog, derivative or fragment is carried by one hybrid vector and sequences from a cDNA or genomic DNA library are carried by the second hybrid vector, transforming yeast host cells with said vectors, isolating the positively transformed cells, and extracting said second hybrid vector to obtain a sequence encoding said ligand.

Similarly, there is also provided a method for isolating and identifying proteins, isoforms, analogs, fragments according to the invention noted above, capable of binding directly to TRAF2 and separately to signalosome components, comprising applying the yeast two-hybrid procedure in which a sequence encoding said TRAF2, is carried by one hybrid vector and sequence from a cDNA or genomic DNA library is carried by the second hybrid vector, the vectors then being used to transform yeast host cells and the positive transformed cells being isolated, followed by extraction of the said second hybrid vector to obtain a sequence encoding a protein which binds to said TRAF2, and retesting the so-identified clones in the same manner using the two-hybrid

assay, for binding to a protein component of the signalosome, and selecting those clones that bind to both.

In yet another aspect of the invention there is provided a method for the modulation or mediation in cells of the activity of NF-κB or by other molecules to which a protein, isoform, analog, fragment or derivative thereof of the invention as noted above, said method comprising treating said cells by introducing into said cells one or more of said protein, isoform, analog, fragment or derivative thereof in a form suitable for intracellular introduction thereof, or introducing into said cells a DNA sequence encoding said one or more protein, isoform, analog, fragment or derivative thereof in the form of a suitable vector carrying said sequence, said vector being capable of effecting the insertion of said sequence into said cells in a way that said sequence is expressed in said cells.

Embodiments of this above method for modulation/mediation in cells of the activity of NF-κB or other molecules include:

(i) A method as above, wherein said treating of cells comprises introducing into said cells a DNA sequence encoding said protein, isoform, fragment, analog or derivative in the form of a suitable vector carrying said sequence, said vector being capable of effecting the insertion of said sequence into said cells in a way that said sequence is expressed in said cells.

(ii) A method as above wherein said treating of said cells is by transfection of said cells with a recombinant animal virus vector comprising the steps of :

(a) constructing a recombinant animal virus vector carrying a sequence encoding a viral surface protein (ligand) that is capable of binding to a specific cell surface receptor on the surface of said cells to be treated and a second

sequence encoding a protein selected from the said protein, isoforms, analogs, fragments and derivatives according to the invention,that when expressed in said cells is capable of modulating/mediating the activity of NF-κB or other said molecules; and

(b) infecting said cells with said vector of (a).

Likewise, the present invention also provides a method for modulating NF-κB modulated/mediated effect on cells comprising treating said cells with the antibodies or active fragments or derivatives thereof, according to the invention as noted above, said treating being by application of a suitable composition containing said antibodies, active fragments or derivatives thereof to said cells, wherein when the signalosome or signalosome-interacting proteins or portions thereof of said cells are exposed on the extracellular surface, said composition is formulated for extracellular application, and when said TRAF2/signalosome and signalosome-interacting proteins are intracellular said composition is formulated for intracellular application.

Other methods of the invention for modulating the NF-κB modulated/mediated effect on cells include :

(i) A method comprising treating said cells with an oligonucleotide sequence encoding an antisense sequence for at least part of the DNA sequence encoding a protein binding to TRAF2 and to a signalosome protein, this DNA sequence being any of the above mentioned ones of the invention, said oligonucleotide sequence being capable of blocking the expression of the TRAF2/signalosome-interacting proteins.

(ii) A method as in (i) above wherein said oligonucleotide sequence is introduced to said cells via a recombinant virus as noted above, wherein said second sequence of said virus encodes said oligonucleotide sequence.

(iii) A method comprising applying the ribozyme procedure in which a vector encoding a ribozyme sequence capable of interacting with a cellular mRNA sequence encoding a TRAF2/signalosome-interacting protein, isoform, analog, fragment or derivative of the invention noted above, is introduced into said cells in a form that permits expression of said ribozyme sequence in said cells, and wherein when said ribozyme sequence is expressed in said cells it interacts with said cellular mRNA sequence and cleaves said mRNA sequence resulting in the inhibition of expression of said TRAF2/signalosome-interacting protein in said cells.

It should be noted that for all the above methods of the invention the protein of the invention as indicated, can be specifically NAP or at least one of the NAP isoforms, analogs, fragments and derivatives thereof.

In the above methods and embodiments thereof of the invention there is included also a method for the prevention or treatment of a pathological condition associated with NF-κB induction or by other molecules to which a protein, isoform, analog, fragment or derivative, according to the invention, binds, said method comprising administering to a patient in need an effective amount of a protein, isoform, analog, fragment or derivative, according to the invention, or a DNA molecule coding therefor, or a molecule capable of disrupting the interaction of said protein, isoform, analog, fragment or derivative, with TRAF2 and/or with an signalosome protein, or any other molecule to which said protein, isoform, analog, fragment or derivative binds. In this method of the invention, said protein of the invention administered to the patient in need can be specifically the protein encoded by clone 10, the protein NAP encoded by clone compl. 10, an isoform, analog, derivative or fragment of NAP, or a DNA molecule coding

therefor. The protein encoded by clone 10 inhibits NF-κB induction, as do other fragments of NAP.

In an additional aspect of the invention there is provided a pharmaceutical composition for the modulation of the TRAF2/signalosome and signalosome interacting protein modulated/mediated effect on cells comprising, as active ingredient an effective amount of NAP, according to the invention, its biologically active fragments, analogs, derivatives or mixtures thereof.

Other pharmaceutical compositions or embodiments thereof according to the invention include :

(i) A pharmaceutical composition for modulating the TRA2F/NF-κB modulated/mediated effect on cells comprising, as active ingredient, a recombinant animal virus vector encoding a protein capable of binding a cell surface receptor and encoding NAP, or an isoform, active fragment or analog thereof, according to the invention.

(ii) A pharmaceutical composition for modulating the TRAF2/NF-κB modulated/mediated effect on cells comprising as active ingredient, an oligonucleotide sequence encoding an anti-sense sequence of the NAP mRNA sequence according to the invention.

A further embodiment of the above pharmaceutical composition is specifically a pharmaceutical composition for the prevention or treatment of a pathological condition associated with NF-κB induction or by other molecules to which a protein, analog, isoform, fragment or derivative, according to the invention binds, said composition comprising an effective amount of a protein, analog, isoform, fragment or derivative, according to the invention or a DNA molecule coding therefor, or a molecule capable of disrupting the interaction of said protein, analog, isoform, fragment or derivative, with TRAF2 and/or with an signalosome protein or

any other molecule to which said protein, analog, isoform, fragment or derivative, binds. In a yet further specific embodiment said pharmaceutical composition comprising an effective amount of the protein encoded by clone 10, or compl. 10, of the NAP protein, an isoform, analog, derivative or fragment of NAP, or a DNA molecule coding therefor.

Known conditions associated with abnormal NF-κB induction include AIDS, autoimmune diseases, and tumors.

Still further aspects and embodiments of the invention are:

(i) A method for identifying and producing a ligand capable of modulating the cellular activity modulated/mediated by TRAF2/signalosome and signalosome interacting protein comprising:

a) screening for a ligand capable of binding to a polypeptide comprising at least a portion of TRAF2 and to amino acids 218 to 416 of NEMO;

b) identifying and characterizing a ligand, other than TRAF2 or NEMO, found by said screening step to be capable of said binding;

c) testing the clones identified in steps (a) and (b) for binding to NEMO and

c) producing said ligand in substantially isolated and purified form.

(ii) A method for identifying and producing a ligand capable of modulating the cellular activity modulated/mediated by a protein, isoform, analog, fragment or derivative, according to the invention, comprising :

a) screening for a ligand capable of binding to a polypeptide comprising at least a portion of the NAP sequence depicted in Fig. 3;

b) identifying and characterizing a ligand, other than TRAF2/NEMO, found by said screening step to be capable of said binding; and

c) producing said ligand in substantially isolated and purified form.

(iii) A method for identifying and producing a ligand capable of modulating the cellular activity modulated/mediated by NAP comprising :

a) screening for a ligand capable of binding to a polypeptide comprising at least a portion of the NAP sequence depicted in Fig. 3;

b) identifying and characterizing a ligand, other than TRAF2 or NEMO, found by said screening step to be capable of said binding; and

c) producing said ligand in substantially isolated and purified form.

(iv) A method for identifying and producing a ligand capable of directly or indirectly modulating the cellular activity modulated/mediated by NAP comprising :

a) screening for a molecule capable of modulating activities modulated/mediated by NAP;

b) identifying and characterizing said molecule; and

c) producing said molecule in substantially isolated and purified form.

(v) A method for identifying and producing a molecule capable of directly or indirectly modulating the cellular activity modulated/mediated by a protein, isoform, analog, fragment or derivative of the invention, comprising :

a) screening for a molecule capable of modulating activities modulated/mediated by a protein, isoform, analog, fragment or derivative according to the invention;

b) identifying and characterizing said molecule; and

c) producing said molecule in substantially isolated and purified form.

Other aspects and embodiments of the present invention are also provided as arising from the following detailed description of the invention.

It should be noted that, where used throughout, the following terms : "modulation/mediation of the TRAF/NF-κB complex component (or TRAF2/NEMO) effect on cells" or any other such "modulation/mediation" mentioned in the specification are understood to encompass *in vitro* as well as *in vivo* treatment and, in addition, also to encompass inhibition or enhancement/augmentation.

All the above and other characteristics and advantages of the invention will be further understood from the following illustrative and non-limitative examples of preferred embodiments thereof.

Brief Description of the Drawings

The present invention will be more clearly understood from the detailed description of the preferred embodiments and from the attached drawings in which:

Fig. 1 shows the nucleotide sequence of clone 10;
Fig. 2 shows the nucleotide sequence of clone compl. 10;
Fig. 3 shows the predicted amino acid sequence of NAP;

Detailed Description of Preferred Embodiments

For purposes of clarity and as an aid in the understanding of the invention, as disclosed and claimed herein, the following terms and abbreviations are defined below:

NF-κB complex (signalosome): within the context of this application, this term is meant to relate to the NF-κB regulatory complex, which is necessary in order to activate NF-κB. Its main components are IKAP, IKK-alpha, IKK-beta, IKK-gamma (NEMO), and optionally, NIK. The complex is reviewed in Scheidereit Nature 395, 225-226, 1998, and references therein.

Functional isoforms, analogs, derivatives and fragments: The term "functional" relates to the ability of such isoforms, derivatives and fragments to bind to a TRAF protein and to a component of the NF-κB complex. In a preferred embodiment of the invention, the TRAF protein is the TRAF2 protein and the component of the NF-κB complex is NEMO.

Biologically active: the term "biologically active" refers to the ability of a protein, isoform, analog, derivative, or fragment thereof to mediate/modulate TRAF2 or NF-κB, i.e., to modulate the effects of a TRAF protein or of the NF-κB complex.

Active: The term "active" in the context of antibody fragments or derivatives (such as single chain antibodies) relates to the ability of the fragment or derivative to retain the binding ability of the said antibody, i.e., the capability to bind to a protein, isoform, fragment, or derivative capable of binding to TRAF and to a component of the NF-κB complex.

A number of methods of the art of molecular biology are not detailed herein, as they are well known to the person of skill in the art. Such methods include site-directed mutagenesis, PCR cloning, phage library screening using oligonucleotide or cDNA probes, expression of cDNAs, analysis of the recombinant proteins, transformation of bacterial and yeast cells, transfection of mammalian cells, and the like. Textbooks describing such methods are e.g., Sambrook et al., Molecular Cloning A Laboratory Manual, Cold Spring Harbor

Laboratory; ISBN: 0879693096, 1989, Current Protocols in Molecular Biology ,by F. M. Ausubel, ISBN: 047150338X, 1988, and Short Protocols in Molecular Biology, by F. M. Ausubel et al. (eds.) 3rd ed. John Wiley & Sons; ISBN: 0471137812, 1995. These publications are incorporated herein in their entirety by reference

In order to identify TRAF2/NF-κB complex interacting proteins and potential substrates, by two hybrid screening method, the two hybrid or three-hybrid system may be used.

The two-hybrid system is used in the method of the invention essentially as described by Fields and Song (Nature 340, p. 245, 1989). Preferably, individual vectors, yeast strains, and libraries may be obtained from Clontech (Palo Alto, USA), as components of the Matchmaker two-hybrid system (#PT1265-1).

The preferred embodiment of the yeast two-hybrid system as used in the method of the invention has been described by Boldin et al., Cell. 85, p. 803-15, 1996. The yeast two-hybrid system has further been described in US Patent 5,580,736, Brent et al. These publications are therefore incorporated herein in their entirety by reference.

The three-hybrid system is used essentially as described by Tirode et al., J. Biol. Chem. 272, p. 22995-9, 1997.

The proteins to be screened with the method of the invention are preferably provided in the form of a cDNA library. However, also genomic libraries or combinatorial libraries may be used. The library is cloned at the C-terminal end of a transcriptional activation domain operable in yeast. Preferably, the transcriptional activation domain of the yeast Gal-4 protein is used, however, a large number of other transcriptional activators may be used. Preferably, the pGAD

GH vector available from Clontech is used for cloning of the library.

The yeast strain used for screening must contain a selection marker such as histidine synthetase, under the control of a promoter that comprises a DNA sequence to which the above-mentioned DNA binding domain binds specifically. Preferably, the yeast cell also contains a reporter gene under the control of a promoter that comprises a DNA sequence to which the above-mentioned DNA binding domain binds specifically. The yeast strain HF7c, available from Clontech, may be used for screening with Gal-4 binding domain hybrids; the strain L40 may be used when lexA is the DNA binding domain used.

After transformation, the yeast cells are placed in conditions selective for active plated onto media lacking certain amino acids, as required for the stability of the plasmids introduced thereinto.

The medium is selective for yeast cells in which the gene for the above-mentioned selection marker is activated. Preferably, the selection marker is the histidine synthetase gene. Yeast cells expressing this gene may be selected for by culturing in medium lacking histidine. Am advantage of this system is the possibility of adding the histidine synthetase inhibitor 3-aminotriazole to the growth medium. It is thus possible to inhibit growth of yeast cells in which a small amount of histidine synthetase is expressed, caused by leaking of the promoter containing the sequence to which the above-mentioned DNA binding domain specifically binds. In some clones, a weak, non-specific interaction between the TRAF and/or NF-κB complex component and the said clone may cause spurious activation of said promoter. Thus, by raising the concentration of said inhibitor in the medium used for selection of interacting clones, it is possible to select only

clones that interact with a certain minimal strength. The concentration of 3-aminotriazole is preferably 7.5 mM.

Clones identified by their ability to grow in medium lacking histidine are further analyzed by quantification of their reporter gene activity. Preferably, the lacZ gene is used a reporter gene. Quantification of lacZ activity is done preferably in liquid culture, as described in Boldin et al., J. Biol. Chem. 270, 7795-8, 1995.

Thus, in one embodiment of the invention, the above-mentioned three-hybrid system is used, wherein in a preferred embodiment thereof, TRAF2 protein is expressed in a bait vector, while the NF-κB complex component is expressed conditionally (e.g., under the control of the Met25 promoter which is positively regulated medium lacking methionine). In this system, clones that require the presence of both TRAF2 and NF-κB complex component for binding to TRAF2 may be detected easily by virtue of comparing the yeast growth in medium with and without methionine.

In another embodiment of the invention, the two-hybrid system is used in a two-step procedure. In a first step, clones that bind to a first bait are isolated. The first bait is preferably a TRAF protein. The so-isolated clones are then tested in a second step for binding to a signalosome component. Clones that bind to both TRAF and to the NF-κB complex (signalosome) component are selected for further study.

Clones that are able to grow in medium lacking histidine and that express lacZ activity are then selected for further study. Firstly, the proteins encoded by the clones are tested for their ability to bind nonrelevant proteins, such as Lamin. In general, Lamin-binding clones are discarded.

Clones that are found to specifically interact with TRAF2/NF-κB complex are then further analyzed. This is done as

well with partial clones as obtained directly from the above-described screening methods, as well as with full-length clones that are obtained on the basis of the sequence of said partial clones. In order to obtain full-length clones, the sequence of the partial clone is obtained by extracting the DNA of said clone from the yeast cells by methods known to the person of skill in the art. The DNA is then transformed into bacteria in order to obtain large amounts of purified DNA, which may be used for sequencing. Alternatively, the insert in the vector, which is preferably the above-noted pGBD vector, may be excised using restriction enzymes and cloned into another vector, such as pBluescript available from Stratagene, for the purpose of sequencing. Sequencing is done y the chain-termination method, preferably using Sequenase2 enzyme as available in the sequencing kit of United States Biochemicals.

The so-obtained sequence may then be entered into a database search program and overlapping sequences are identified by computer search. The programs used are well known to all of skill in the art and comprise e.g., the GCG (genetics computer group) package. Preferably, a search utility such as Basic Local Alignment Search Tool (BLAST) available from the EMBL server (e.g., http://dove.embl-heidelberg.de/Blast2/) is used. The Blastn command may be used for searching for nucleotide sequences that are overlapping or similar with the clone identified.

The protein identified by the method of the invention is provided as a fusion protein with a DNA binding domain. Therefore, the frame in which the nucleic acid sequence should be translated, is known, as it must be in-frame with the coding sequence of the DNA binding domain. The DNA sequence of the clone identified by the invention can therefore be unambiguously translated into amino acid sequence. The Blastp program, available on the above-noted

EMBL server, may then be used for identification of overlapping protein sequences or similar proteins.

Alternatively, or in addition to the above-noted methods of searching databases, a library, such as a genomic library or a cDNA library, may be screened in order to identify complete clones. Such screening methods are described in the above-noted Sambrook et al and Ausubel et al. Alternatively, or in addition, PCR-based cloning techniques may be used, such as rapid amplification of cDNA ends (5' and 3' RACE, Graham et al., Biochem Biophys Res Commun 177, p. 8-16, 1991, and references therein).

The partial clones identified in the screening assay of the invention, or the full-length clones obtained by any of the above methods, are then further investigated. This is done e.g., by testing the ability of the clones to modulate NF-κB activity and/or induction. In one embodiment, the DNA sequence of the clone is transferred to a mammalian expression vector and the so-obtained construct transfected into a cell that contains an NF-κB reactive promoter driving a reporter gene. The expression vector for expression of the clone preferably comprises a strong promoter for expression of the clone and of the second protein, such as the Rous sarcoma virus (RSV, Yamamoto et al. Cell 22, p. 787-97, 1980), myeloproliferative sarcoma virus (MPSV, Artelt P et al., Gene 68 p. 213-9, 1988), Cytomegalovirus (CMV, Thomsen, et al. PNAS 81 p. 659-63, 1984), or similar promoters of viral or cellular origin. Preferred NF-κB reactive promoters include the CMV promoter, the HIV promoter, and a promoter selected from the group of Immunoglobulin gene promoters. Most preferred is the HIV promoter. Preferred reporter genes include green fluorescent protein, lacZ, CAT, human growth hormone, and luciferase.

NF-κB activity may be induced in this system and the influence of the clone on the induction measured using the

reporter gene. Inducers of NF-κB include TNF, overexpression of TRAF, TNF receptor or intracellular domains thereof, or the like inducers as known in the art.

The present invention relates to a DNA sequence coding for proteins interacting with TRAF2 and the signalosome proteins.

Moreover, the present invention further concerns the DNA sequences encoding a biologically active isoform, allelic variant, fragment, functional analog, mutant or derivative of the TRAF2/NF-κB complex interacting protein, and the protein, isoform, allelic variant, fragment, functional analog, mutant or derivative encoded thereby. The preparation of such analogs, fragments, mutants and derivatives is by standard procedure (see for example, Sambrook et al., 1989) in which in the DNA sequences encoding the TRAF2/NF-κB complex interacting protein, one or more codons may be deleted, added or substituted by another, to yield analogs having at least one amino acid residue change with respect to the native protein.

Of the above DNA sequences of the invention which encode a TRAF2/NF-κB complex interacting protein, isoform, allelic variant, fragment, functional analog, mutant or derivative, there is also included, as an embodiment of the invention, DNA sequences capable of hybridizing with a cDNA sequence derived from the coding region of a native TRAF2/NF-κB complex interacting protein, in which such hybridization is performed under moderately stringent conditions, and which hybridizable DNA sequences encode a biologically active TRAF2/NF-κB complex interacting protein. These hybridizable DNA sequences therefore include DNA sequences which have a relatively high homology to the native TRAF2/NF-κB complex interacting protein cDNA sequence and as such represent TRAF2/NF-κB complex interacting protein-like sequences which

:or example, naturally-derived sequences encoding the 'RAF2/NF-κB complex interacting protein isoforms, or -occurring sequences encoding proteins belonging to a f TRAF2/NF-κB complex interacting protein-like encoding a protein having the activity of TRAF2/NF-x interacting protein. Further, these sequences may 'or example, include non-naturally occurring, ally produced sequences, that are similar to the :AF2/NF-κB complex interacting protein cDNA sequence rporate a number of desired modifications. Such sequences therefore include all of the possible encoding analogs, fragments and derivatives of κB complex interacting protein, all of which have the of TRAF2/NF-κB complex interacting protein.

To obtain the various above noted naturally TRAF2/NF-κB complex interacting protein-like , standard procedures of screening and isolation of -derived DNA or RNA samples from various tissues may 'ed using the natural TRAF2/NF-κB complex interacting :DNA or portion thereof as probe (see for example procedures set forth in Sambrook et al., 1989).

The invention relates to a TRAF2/NF-κB complex ng protein as may be identified by the above assay. The invention also relates to a polypeptide .n substantially corresponding to TRAF2/NF-κB complex ng protein. The term "substantially corresponding" not only TRAF2/NF-κB complex interacting protein but peptides or proteins that are analogs thereof.

Analogs that substantially correspond to TRAF2/NF-κB :nteracting protein are those polypeptides in which)re amino acid of the TRAF2/NF-κB complex interacting

protein's amino acid sequence has been replaced with another amino acid, deleted and/or inserted, provided that the resulting protein exhibits substantially the same or higher biological activity as the TRAF2/NF-κB complex interacting protein to which it corresponds.

In order to substantially correspond to TRAF2/NF-κB complex interacting protein, the changes in the sequence of TRAF2/NF-κB complex interacting proteins, such as isoforms are generally relatively minor. Although the number of changes may be more than ten, preferably there are no more than ten changes, more preferably no more than five, and most preferably no more than three such changes. While any technique can be used to find potentially biologically active proteins which substantially correspond to TRAF2/NF-κB complex interacting proteins, one such technique is the use of conventional mutagenesis techniques on the DNA encoding the protein, resulting in a few modifications. The proteins expressed by such clones can then be screened for their ability to bind to TRAF2/NF-κB complex and to modulate TRAF/NF-κB complex activity in modulation/mediation of the intracellular pathways noted above.

"Conservative" changes are those changes which would not be expected to change the activity of the protein and are usually the first to be screened as these would not be expected to substantially change the size, charge or configuration of the protein and thus would not be expected to change the biological properties thereof.

Conservative substitutions of TRAF2/NF-κB complex interacting proteins include an analog wherein at least one amino acid residue in the polypeptide has been conservatively replaced by a different amino acid. Such substitutions preferably are made in accordance with the following list as

presented in Table IA, which substitutions may be determined by routine experimentation to provide modified structural and functional properties of a synthesized polypeptide molecule while maintaining the biological activity characteristic of TRAF-2/NF-κB complex interacting protein.

Table IA

Original Residue	Exemplary Substitution
Ala	Gly;Ser
Arg	Lys
Asn	Gln;His
Asp	Glu
Cys	Ser
Gln	Asn
Glu	Asp
Gly	Ala;Pro
His	Asn;Gln
Ile	Leu;Val
Leu	Ile;Val
Lys	Arg;Gln;Glu
Met	Leu;Tyr;Ile

Phe	Met;Leu;Tyr
Ser	Thr
Thr	Ser
Trp	Tyr
Tyr	Trp;Phe
Val	Ile;Leu

Alternatively, another group of substitutions of TRAF2/NF-κB complex interacting protein are those in which at least one amino acid residue in the polypeptide has been removed and a different residue inserted in its place according to the following Table IB. The types of substitutions which may be made in the polypeptide may be based on analysis of the frequencies of amino acid changes between a homologous protein of different species, such as those presented in Table 1-2 of Schulz et al., G.E., Principles of Protein Structure Springer-Verlag, New York, NY, 1798, and Figs. 3-9 of Creighton, *T.E.*, Proteins: Structure and Molecular Properties, W.H. Freeman & Co., San Francisco, CA 1983. Based on such an analysis, alternative conservative substitutions are defined herein as exchanges within one of the following five groups:

<u>TABLE IB</u>

1. Small aliphatic, nonpolar or slightly polar residues: Ala, Ser, Thr (Pro, Gly);

2. Polar negatively charged residues and their amides: Asp, Asn, Glu, Gln;

3. Polar, positively charged residues:

His, Arg, Lys;

4. Large aliphatic nonpolar residues:

Met, Leu, Ile, Val (Cys); and

5. Large aromatic residues: Phe, Tyr, Trp.

The three amino acid residues in parentheses above have special roles in protein architecture. Gly is the only residue lacking any side chain and thus imparts flexibility to the chain. This however tends to promote the formation of secondary structure other than a-helical. Pro, because of its unusual geometry, tightly constrains the chain and generally tends to promote beta-turn-like structures, although in some cases Cys can be capable of participating in disulfide bond formation which is important in protein folding. Note that Schulz *et al.*, *supra*, would merge Groups 1 and 2, above. Note also that Tyr, because of its hydrogen bonding potential, has significant kinship with Ser, and Thr, etc.

Conservative amino acid substitutions according to the present invention, e.g., as presented above, are known in the art and would be expected to maintain biological and structural properties of the polypeptide after amino acid substitution. Most deletions and substitutions according to the present invention are those which do not produce radical changes in the characteristics of the protein or polypeptide molecule. "Characteristics" is defined in a non-inclusive manner to define both changes in secondary structure, e.g. a-helix or beta-sheet, as well as changes in biological activity, e.g., binding to TRAF2/NF-κB complex and/or mediation of the effect of TRAF2/NF-κB complex on cell death.

Examples of production of amino acid substitutions in proteins which can be used for obtaining analogs of TRAF2/NF-κB complex interacting proteins for use in the present invention include any known method steps, such as presented in U.S. patent RE 33,653, 4,959,314, 4,588,585 and 4,737,462, to Mark et al.; 5,116,943 to Koths et al., 4,965,195 to Namen et al.; 4,879,111 to Chong et al.; and 5,017,691 to Lee et al.; and lysine substituted proteins presented in U.S. patent No. 4,904,584 (Shaw et al.).

Besides conservative substitutions discussed above which would not significantly change the activity of TRAF2/NF-κB complex interacting protein, either conservative substitutions or less conservative and more random changes, which lead to an increase in biological activity of the analogs of TRAF2/NF-κB complex interacting proteins, are intended to be within the scope of the invention.

When the exact effect of the substitution or deletion is to be confirmed, one skilled in the art will appreciate that the effect of the substitution(s), deletion(s), etc., will be evaluated by routine binding and cell death assays. Screening using such a standard test does not involve undue experimentation.

Acceptable TRAF2/NF-κB complex interacting analogs are those which retain at least the capability of interacting with TRAF2/NF-κB complex, and thereby, mediate the activity of TRAF2/NF-κB complex in the intracellular pathways, or modulate the activity of TRA2F/NF-κB complex itself. In such a way, analogs can be produced which have a so-called dominant-negative effect, namely, an analog which is defective either in binding to TRAF2/NF-κB complex, or in subsequent signaling or other activity following such binding. Such analogs can be used, for example, to

inhibit the NF-κB inducing effect of TRAF proteins, e.g., of TRAF-2.

At the genetic level, these analogs are generally prepared by site-directed mutagenesis of nucleotides in the DNA encoding the TRAF2/NF-κB complex interacting protein, thereby producing DNA encoding the analog, and thereafter synthesizing the DNA and expressing the polypeptide in recombinant cell culture. The analogs typically exhibit the same or increased qualitative biological activity as the naturally occurring protein, Ausubel *et al.*, Current Protocols in Molecular Biology, Greene Publications *and Wiley Interscience, New York, NY*, 1987-1995; Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989.

Preparation of a TRAF2/NF-κB complex interacting protein in accordance herewith, or an alternative nucleotide sequence encoding the same polypeptide but differing from the natural sequence due to changes permitted by the known degeneracy of the genetic code, can be achieved by site-specific mutagenesis of DNA that encodes an earlier prepared analog or a native version of a TRAF2/NF-κB complex interacting protein. Site-specific mutagenesis allows the production of analogs through the use of specific oligonucleotide sequences that encode the DNA sequence of the desired mutation, as well as a sufficient number of adjacent nucleotides, to provide a primer sequence of sufficient size and sequence complexity to form a stable duplex on both sides of the deletion junction being traversed. Typically, a primer of about 20 to 25 nucleotides in length is preferred, with about 5 to 10 complementing nucleotides on each side of the sequence being altered. In general, the technique of site-specific mutagenesis is well known in the art, as exemplified by publications such as Adelman *et al.*, *DNA* 2:183 (1983), the disclosure of which is incorporated herein by reference.

As will be appreciated, the site-specific mutagenesis technique typically employs a phage vector that exists in both a single-stranded and double-stranded form. Typical vectors useful in site-directed mutagenesis include vectors such as the M13 phage, for example, as disclosed by Messing *et al.*, *Third Cleveland Symposium on Macromolecules and Recombinant DNA*, Editor A. Walton, Elsevier, Amsterdam (1981), the disclosure of which is incorporated herein by reference. These phage are readily available commercially and their use is generally well known to those skilled in the art. Alternatively, plasmid vectors that contain a single-stranded phage origin of replication (Veira *et al.*, *Meth. Enzymol.* 153:3, 1987) may be employed to obtain single-stranded DNA.

In general, site-directed mutagenesis in accordance herewith is performed by first obtaining a single-stranded vector that includes within its sequence a DNA sequence that encodes the relevant polypeptide. An oligonucleotide primer bearing the desired mutated sequence is prepared synthetically by automated DNA/oligonucleotide synthesis. This primer is then annealed with the single-stranded protein-sequence-containing vector, and subjected to DNA-polymerizing enzymes such as *E. coli* polymerase I Klenow fragment, to complete the synthesis of the mutation-bearing strand. Thus, a mutated sequence and the second strand bears the desired mutation. This heteroduplex vector is then used to transform appropriate cells, such as *E. coli* JM101 cells, and clones are selected that include recombinant vectors bearing the mutated sequence arrangement.

After such a clone is selected, the mutated 2/NF-κB complex interacting protein sequence may be removed and placed in an appropriate vector, generally a transfer or expression vector of the type that may be employed for transfection of an appropriate host.

Accordingly, gene or nucleic acid encoding for a TRAF2/NF-κB complex interacting protein can also be detected, obtained and/or modified, *in vitro*, *in situ* and/or *in vivo*, by the use of known DNA or RNA amplification techniques, such as PCR and chemical oligonucleotide synthesis. PCR allows for the amplification (increase in number) of specific DNA sequences by repeated DNA polymerase reactions. This reaction can be used as a replacement for cloning; all that is required is a knowledge of the nucleic acid sequence. In order to carry out PCR, primers are designed which are complementary to the sequence of interest. The primers are then generated by automated DNA synthesis. Because primers can be designed to hybridize to any part of the gene, conditions can be created such that mismatches in complementary base pairing can be tolerated. Amplification of these mismatched regions can lead to the synthesis of a mutagenized product resulting in the generation of a peptide with new properties (i.e., site directed mutagenesis). See also, e.g., Ausubel, *supra*, Ch. 16. Also, by coupling complementary DNA (cDNA) synthesis, using reverse transcriptase, with PCR, RNA can be used as the starting material for the synthesis of the extracellular domain of a prolactin receptor without cloning.

Furthermore, PCR primers can be designed to incorporate new restriction sites or other features such as termination codons at the ends of the gene segment to be amplified. This placement of restriction sites at the 5' and 3' ends of the amplified gene sequence allows for gene segments encoding TRAF2/NF-κB complex interacting protein or a fragment thereof to be custom designed for ligation other sequences and/or cloning sites in vectors.

PCR and other methods of amplification of RNA and/or DNA are well known in the art and can be used according to the present invention without undue experimentation, based on the teaching and guidance presented herein. Known methods of DNA or

RNA amplification include, but are not limited to polymerase chain reaction (PCR) and related amplification processes (see, e.g., U.S. patent Nos. 4,683,195, 4,683,202, 4,800,159, 4,965,188, to Mullis *et al.*; 4,795,699 and 4,921,794 to Tabor *et al.*; 5,142,033 to Innis; 5,122,464 to Wilson *et al.*; 5,091,310 to Innis; 5,066,584 to Gyllensten *et al.*; 4,889,818 to Gelfand *et al.*; 4,994,370 to Silver *et al.*; 4,766,067 to Biswas; 4,656,134 to Ringold; and Innis *et al.*, eds., *PCR Protocols: A Guide to Method and Applications*) and RNA mediated amplification which uses anti-sense RNA to the target sequence as a template for double stranded DNA synthesis (U.S. patent No. 5,130,238 to Malek *et al.*, with the tradename NASBA); and immuno-PCR which combines the use of DNA amplification with antibody labeling (Ruzicka *et al.*, *Science* 260:487 (1993); Sano *et al.*, *Science* 258:120 (1992); Sano *et al.*, *Biotechniques* 9:1378 (1991)), the entire contents of which patents and reference are entirely incorporated herein by reference.

In an analogous fashion, biologically active fragments of TRAF2/NF-κB complex interacting proteins (e.g. those of any of the TRAF2/NF-κB complex interacting proteins or its isoforms) may be prepared as noted above with respect to the analogs of TRAF2/NF-κB complex interacting protein. Suitable fragments of TRAF2/NF-κB complex interacting protein are those which retain the TRAF2/NF-κB complex interacting protein capability and which can mediate the biological activity of TRAF proteins and/or or the NF-κB complex or of other proteins associated therewith directly or indirectly. Accordingly, TRAF2/NF-κB complex interacting protein fragments can be prepared which have a dominant-negative or a dominant-positive effect as noted above with respect to the analogs. It should be noted that these fragments represent a special class of the analogs of the invention, namely, they are defined portions of TRAF2/NF-κB

complex interacting proteins derived from the full TRAF2/NF-κB complex interacting protein sequence (e.g., from that of any one of the TRAF2/NF-κB complex interacting protein or its isoforms), each such portion or fragment having any of the above-noted desired activities. Such fragment may be, e.g., a peptide.

Similarly, derivatives may be prepared by standard modifications of the side groups of one or more amino acid residues of the TRAF2/NF-κB complex interacting protein, its analogs or fragments, or by conjugation of the TRAF2/NF-κB complex interacting protein, its analogs or fragments, to another molecule e.g. an antibody, enzyme, receptor, etc., as are well known in the art. Accordingly, "derivatives" as used herein covers derivatives which may be prepared from the functional groups which occur as side chains on the residues or the N- or C-terminal groups, by means known in the art, and are included in the invention. Derivatives may have chemical moieties such as carbohydrate or phosphate residues, provided such a fraction has the same or higher biological activity as TRAF2/NF-κB complex interacting proteins.

For example, derivatives may include aliphatic esters of the carboxyl groups, amides of the carboxyl groups by reaction with ammonia or with primary or secondary amines, N-acyl derivatives or free amino groups of the amino acid residues formed with acyl moieties (e.g., alkanoyl or carbocyclic aroyl groups) or O-acyl derivatives of free hydroxyl group (for example that of seryl or threonyl residues) formed with acyl moieties.

The term "derivatives" is intended to include only those derivatives that do not change one amino acid to another of the twenty commonly occurring natural amino acids.

Expression of a protein or peptide in a mammalian cell may be done by inserting the DNA coding for the protein to be tested into a vector comprising a promoter, optionally an intron sequence and splicing donor/acceptor signals, and

further optionally comprising a termination sequence. These techniques are in general described in the above-noted Current Protocols, chapter 16.

The above promoter, intron, and termination sequences are operable in mammalian cells. The promoter is preferably a strong promoter such as the above-noted RSV, CMV, or MPSV promoter. The promoter may also be the SV40 early promoter (Everett, et al. Nucleic Acids Res. 11 p. 2447-64, 1983, and references therein), or a cellular promoter, such as the beta-actin promoter or the ELF-1 promoter (Tokushige, et al., J Virol Methods. 64 p. 73-80, 1997). Also, a hybrid promoter may be used, such as the hybrid between the lac operator and the human ELF-1 alpha promoter as described by Edamatsu et al. (Gene 187,p. 289-94, 1997), the CMV-beta actin hybrid promoter described by Akagi et al., Kidney Int. 51, p. 1265-9, 1997), or the hybrid between tet operator sequences and the CMV promoter (Furth et al., PNAS 91, p. 9302-6, 1994, and references therein).

Intron sequences which may be inserted as complete sequences, i.e., including the splice donor and acceptor sites, may be inserted into the coding sequence of the protein which it is desired to express. Insertion if such intron sequences may enhance RNA stability and thus enhance production of the desired protein. While in principle, suitable intron sequences may be selected from any gene containing introns, preferred intron sequences are the beta-actin intron, the SV 40 intron, and the p55 TNF receptor intron.

The intron sequence may contain enhancer elements which may enhance transcription from the above-noted promoters.

Often, intron sequences also contain transcriptional or translational control sequences that confer tissue specific

expression. Therefore, when it is desired to express a protein of the invention in a tissue-specific manner, such intron sequences may be advantageously employed. An example of an intron containing tissue-specific enhancer elements is the erythroid-specific enhancer located in intron 8 of the human 5-aminolevulinate synthase 2 gene (Surinya et al. J Biol Chem. 273, p. 16798-809, 1998), and a discussion of the principle of enhancing protein production using intron sequences, together with example intron sequences, is provided in Huang et al. Nucleic Acids Res. 18, p. 937-47, 1990).

Transcriptional termination sequences and polyadenylation signals may be added at the 3' end of the DNA coding for the protein that it is desired to express. Such sequences may be found in many or even most genes. Advantageously, the SV 40 polyadenylation signal is used (Schek et al., Mol Cell Biol., p. 5386-93, 1992, and references therein).

A preferred vector for expression of a protein in a mammalian cell is the pcDNAHis vector (Invitrogen) which contains the CMV promoter for driving expression of the gene encoding the desired protein. Other vectors that may be used include the pCDNA3 or pMPSVEH vectors. These vectors contain the CMV and the MPSV promoters, respectively.

Using recombinant expression of the protein to be tested, said protein can now be evaluated for its effect on the NF-κB modulating signals which are mediated e.g., by TRAF proteins. To that end, NF-κB may be induced in a variety of ways as known in the art, e.g., by treatment with TNF or IL-1 in cells responsive to such treatment by NF-κB modulation. This may also be achieved by overexpression of an NF-κB inducing proteins, e.g., the CD120a intracellular domain, the CD95 intracellular domain, or the like proteins. Receptor activation may either be achieved by contacting the receptors

with ligand or by cross-linking receptors with antibodies, preferably polyclonal antibodies (see Engelmann et al. J. Biol. Chem. 265, p. 14497-504, 1990).

The mammalian cells are preferably HeLa or human embryonic kidney (HEK) 293-T cells. The transfection is preferably done by the calcium phosphate method as described in the above Current Protocols. The morphology of the cells if evaluated one to 150 hours after transfection, preferably 4 to 35 hours and most preferably 20 hours after transfection.

Generation of antibodies

Polyclonal antibodies may be generated in rabbits, chicken, mice, rats, sheep, or similar mammals. For generation of antibodies against a protein or peptide of the invention, the protein or peptide is produced, as described above, by recombinant DNA technology in mammalian cells. The protein may also be produced in bacterial or insect cells as detailed in the above-noted Current Protocols, chapter 16.

The protein or peptide is purified from the cells in which it has bee produced. Protein purification methods are known to the person of skill in the art and are detailed e.g., in the above-noted Current Protocols in Molecular Biology, chapter 16, and in Current Protocols in Protein Science, Wiley and Sons Inc. chapters 5 and 6. Advantageously, the protein may be produced as a fusion with a second protein, such as Glutathione-S-transferase or the like, or a sequence tag, such as the histidine tag sequence. The use of fusion or tagged proteins simplifies the purification procedure, as detailed in the above-noted Current Protocols in Molecular Biology, chapter 16, and in the instructions for the commercially available Qiagen (Qiagen GmbH 40724 Hilden Germany) his-tag protein expression and purification kit.

If the protein or peptide has been expressed as a fusion protein, it is desirable to cleave the fusion partner before using the protein for the generation of antibodies, in order to avoid generation of antibodies against the fusion partner. The cleavage of fusion partners and the isolation of the desired protein is described in the above-noted Current Protocols in molecular Biology, chapter 16. Vectors, protocols and reagents for expressing and purifying maltose-binding protein fused recombinant proteins are also available commercially.

When producing a peptide of the invention, it may be desirable not to remove the fusion partner, as the fusion protein may stimulate the production of antibodies against the peptide. Generally this consideration will be relevant when generating antibodies from peptides that are less than 50 amino acids in length.

As noted further above, peptide may also be synthesized by chemical methods known in the art of chemistry.

The generation of polyclonal antibodies against proteins is described chapter 2 of Current Protocols in Immunology, Wiley and Sons Inc. The generation of antibodies against peptides may necessitate some changes in protocol, because of the generally lower antigenicity of peptides when compared to proteins. The generation of polyclonal antibodies against peptides is described in the above-noted Current Protocols in Immunology, chapter 9.

Monoclonal antibodies may be prepared from B cells taken from the spleen or lymphnodes of immunized animals, in particular rats or mice, by fusion with immortalized B cells under conditions which favor the growth of hybrid cells. For fusion of murine B cells, the cell line Ag-8 is preferred.

The technique of generating monoclonal antibodies is described in many articles and textbooks, such as the above-

noted chapter 2 of Current Protocols in Immunology. Chapter 9 therein describes the immunization, with peptides, or animals. Spleen or lymphnode cells of these animals may be used in the same way as spleen or lymphnode cells of protein-immunized animals, for the generation of monoclonal antibodies as described in chapter 2 therein.

The techniques used in generating monoclonal antibodies are further described in Kohler and Milstein, Nature 256, 495-497, and in USP 4,376,110.

The preparation of antibodies from a gene bank of human antibodies the hypervariable regions thereof are replaced by almost random sequences, is described in USP 5,840,479. Such antibodies are preferred if it is difficult to immunize an animal with a given peptide or protein. Some structures are poorly immunogenic and may remain so despite of the addition of adjuvants and of linking to other proteins in fusion constructs. The antibodies described in USP 5,840,479 are further preferred if it is desired to use antibodies with a structure similar to human antibodies, for instance, when antibodies are desired that have a low immunogenicity in humans.

Once a suitable antibody has been identified, it may be desired to change the properties thereof. For instance, a chimeric antibody may achieve higher yields in production. Chimeric antibodies wherein the constant regions are replaced with constant regions of human antibodies are further desired when it is desired that the antibody be of low immunogenicity in humans. The generation of chimeric antibodies is described in a number of publications, such as Cabilly et al., PNAS 81, p. 3273, 1984, Morrison et al., PNAS 81, 6851, 1984, Boulianne et al, Nature 312, p. 643, 1984, EP 125023, EP 171496, EP 173494, EP 184187, WO 86/01533, WO 87/02671, and Harlow and

Lane, Antibodies: A Laboratory Manual, Cold Spring harbor Laboratory, 1988.

Another type of antibody is an anti-idiotypic antibody. An anti-idiotypic (anti-Id) antibody is an antibody which recognizes unique determinants generally associated with the antigen-binding site of an antibody. An Id antibody can be prepared by immunizing an animal of the same species and genetic type (e.g. mouse strain) as the source of the mAb to which an anti-Id is being prepared. The immunized animal will recognize and respond to the idiotypic determinants of the immunizing antibody by producing an antibody to these idiotypic determinants (the anti-Id antibody). See, for example, U.S. Patent No. 4,699,880, which is herein entirely incorporated by reference.

The anti-Id antibody may also be used as an "immunogen" to induce an immune response in yet another animal, producing a so-called anti-anti-Id antibody. The anti-anti-Id may be epitopically identical to the original mAb which induced the anti-Id. Thus, by using antibodies to the idiotypic determinants of a mAb, it is possible to identify other clones expressing antibodies of identical specificity.

Accordingly, mAbs generated against the TRAF/NF-κB complex interacting protein, analogs, fragments or derivatives thereof, of the present invention may be used to induce anti-Id antibodies in suitable animals, such as BALB/c mice. Spleen cells from such immunized mice are used to produce anti-Id hybridomas secreting anti-Id mAbs. Further, the anti-Id mAbs can be coupled to a carrier such as keyhole limpet hemocyanin (KLH) and used to immunize additional BALB/c mice. Sera from these mice will contain anti-anti-Id antibodies that have the binding properties of the original mAb specific for an epitope of the above TRAF/NF-κB complex interacting protein, or analogs, fragments and derivatives thereof.

The anti-Id mAbs thus have their own idiotypic epitopes, or "idiotopes" structurally similar to the epitope being evaluated.

The term "antibody" is also meant to include both intact molecules as well as fragments thereof, such as, for example, Fab and F(ab')2, which are capable of binding antigen. Fab and F(ab')2 fragments lack the Fc fragment of intact antibody, clear more rapidly from the circulation, and may have less non-specific tissue binding than an intact antibody (Wahl et al., *J. Nucl. Med.* 24:316-325 (1983)).

It will be appreciated that Fab and F(ab')2 and other fragments of the antibodies useful in the present invention may be used for the detection and quantitation of the TRAF/NF-κB complex interacting protein according to the methods disclosed herein for intact antibody molecules. Such fragments are typically produced by proteolytic cleavage, using enzymes such as papain (to produce Fab fragments) or pepsin (to produce F(ab')2 fragments).

An antibody is said to be "capable of binding" a molecule if it is capable of specifically reacting with the molecule to thereby bind the molecule to the antibody. The term "epitope" is meant to refer to that portion of any molecule capable of being bound by an antibody which can also be recognized by that antibody. Epitopes or "antigenic determinants" usually consist of chemically active surface groupings of molecules such as amino acids or sugar side chains and have specific three dimensional structural characteristics as well as specific charge characteristics.

An "antigen" is a molecule or a portion of a molecule capable of being bound by an antibody which is additionally capable of inducing an animal to produce antibody capable of binding to an epitope of that antigen. An antigen may have one or more than one epitope. The specific reaction referred to

above is meant to indicate that the antigen will react, in a highly selective manner, with its corresponding antibody and not with the multitude of other antibodies which may be evoked by other antigens.

The antibodies, including fragments of antibodies, useful in the present invention may be used to quantitatively or qualitatively detect the TRAF2/NF-κB complex interacting protein in a sample or to detect presence of cells which express the TRAF2/NF-κB complex interacting protein of the present invention. This can be accomplished by immunofluorescence techniques employing a fluorescently labeled antibody (see below) coupled with light microscopic, flow cytometric, or fluorometric detection.

The antibodies (or fragments thereof) useful in the present invention may be employed histologically, as in immunofluorescence or immunoelectron microscopy, for *in situ* detection of the TRAF2/NF-κB complex interacting protein of the present invention. *In situ* detection may be accomplished by removing a histological specimen from a patient, and providing the labeled antibody of the present invention to such a specimen. The antibody (or fragment) is preferably provided by applying or by overlaying the labeled antibody (or fragment) to a biological sample. Through the use of such a procedure, it is possible to determine not only the presence of the TRAF2/NF-κB complex interacting protein, but also its distribution on the examined tissue. Using the present invention, those of ordinary skill will readily perceive that any of wide variety of histological methods (such as staining procedures) can be modified in order to achieve such *in situ* detection.

Such assays for the TRAF2/NF-κB complex interacting protein of the present invention typically comprises incubating a biological sample, such as a biological fluid, a tissue extract, freshly harvested cells such as lymphocytes or leukocytes, or

cells which have been incubated in tissue culture, in the presence of a detectably labeled antibody capable of identifying the TRAF2/NF-κB complex interacting protein, and detecting the antibody by any of a number of techniques well known in the art.

The biological sample may be treated with a solid phase support or carrier such as nitrocellulose, or other solid support or carrier which is capable of immobilizing cells, cell particles or soluble proteins. The support or carrier may then be washed with suitable buffers followed by treatment with a detectably labeled antibody in accordance with the present invention, as noted above. The solid phase support or carrier may then be washed with the buffer a second time to remove unbound antibody. The amount of bound label on said solid support or carrier may then be detected by conventional means.

By "solid phase support", "solid phase carrier", "solid support", "solid carrier", "support" or "carrier" is intended any support or carrier capable of binding antigen or antibodies. Well-known supports or carriers, include glass, polystyrene, polypropylene, polyethylene, dextran, nylon amylases, natural and modified celluloses, polyacrylamides, gabbros and magnetite. The nature of the carrier can be either soluble to some extent or insoluble for the purposes of the present invention. The support material may have virtually any possible structural configuration so long as the coupled molecule is capable of binding to an antigen or antibody. Thus, the support or carrier configuration may be spherical, as in a bead, cylindrical, as in the inside surface of a test tube, or the external surface of a rod. Alternatively, the surface may be flat such as a sheet, test strip, etc. Preferred supports or carriers include polystyrene beads. Those skilled in the art will know may other suitable carriers for binding antibody or antigen, or will be able to ascertain the same by use of routine experimentation.

The binding activity of a given lot of antibody, of the invention as noted above, may be determined according to well known methods. Those skilled in the art will be able to determine operative and optimal assay conditions for each determination by employing routine experimentation.

Other such steps as washing, stirring, shaking, filtering and the like may be added to the assays as is customary or necessary for the particular situation.

One of the ways in which an antibody in accordance with the present invention can be detectably labeled is by linking the same to an enzyme and used in an enzyme immunoassay (EIA). This enzyme, in turn, when later exposed to an appropriate substrate, will react with the substrate in such a manner as to produce a chemical moiety which can be detected, for example, by spectrophotometric, fluorometric or by visual means. Enzymes which can be used to detectably label the antibody include, but are not limited to, malate dehydrogenase, staphylococcal nuclease, delta-5-steroid isomerase, yeast alcohol dehydrogenase, alpha-glycerophosphate dehydrogenase, triose phosphate isomerase, horseradish peroxidase, alkaline phosphatase, asparaginase, glucose oxidase, beta-galactosidase, ribonuclease, urease, catalase, glucose-6-phosphate dehydrogenase, glucoamylase and acetylcholin-esterase. The detection can be accomplished by colorimetric methods which employ a chromogenic substrate for the enzyme. Detection may also be accomplished by visual comparison of the extent of enzymatic reaction of a substrate in comparison with similarly prepared standards.

Detection may be accomplished using any of a variety of other immunoassays. For example, by radioactive labeling the antibodies or antibody fragments, it is possible to detect R-PTPase through the use of a radioimmunoassay (RIA). A good description of RIA may be found in Laboratory Techniques and Biochemistry in Molecular Biology, by Work, T.S. et al., North

Holland Publishing Company, NY (1978) with particular reference to the chapter entitled "An Introduction to Radioimmune Assay and Related Techniques" by Chard, T., incorporated by reference herein. The radioactive isotope can be detected by such means as the use of a g counter or a scintillation counter or by autoradiography.

It is also possible to label an antibody in accordance with the present invention with a fluorescent compound. When the fluorescently labeled antibody is exposed to light of the proper wavelength, its presence can be then detected due to fluorescence. Among the most commonly used fluorescent labeling compounds are fluorescein isothiocyanate, rhodamine, phycoerythrine, pycocyanin, allophycocyanin, o-phthaldehyde and fluorescamine.

The antibody can also be detectably labeled using fluorescence emitting metals such as ^{152}E, or others of the lanthanide series. These metals can be attached to the antibody using such metal chelating groups as diethylenetriamine pentaacetic acid (ETPA).

The antibody can also be detectably labeled by coupling it to a chemiluminescent compound. The presence of the chemiluminescent-tagged antibody is then determined by detecting the presence of luminescence that arises during the course of a chemical reaction. Examples of particularly useful chemiluminescent labeling compounds are luminol, isoluminol, theromatic acridinium ester, imidazole, acridinium salt and oxalate ester.

Likewise, a bioluminescent compound may be used to label the antibody of the present invention. Bioluminescence is a type of chemiluminescence found in biological systems in which a catalytic protein increases the efficiency of the chemiluminescent reaction. The presence of a bioluminescent protein is determined by detecting the presence of luminescence.

Important bioluminescent compounds for purposes of labeling are luciferin, luciferase and aequorin.

An antibody molecule of the present invention may be adapted for utilization in an immunometric assay, also known as a "two-site" or "sandwich" assay. In a typical immunometric assay, a quantity of unlabeled antibody (or fragment of antibody) is bound to a solid support or carrier and a quantity of detectably labeled soluble antibody is added to permit detection and/or quantitation of the ternary complex formed between solid-phase antibody, antigen, and labeled antibody.

Typical, and preferred, immunometric assays include "forward" assays in which the antibody bound to the solid phase is first contacted with the sample being tested to extract the antigen from the sample by formation of a binary solid phase antibody-antigen complex. After a suitable incubation period, the solid support or carrier is washed to remove the residue of the fluid sample, including unreacted antigen, if any, and then contacted with the solution containing an unknown quantity of labeled antibody (which functions as a "reporter molecule"). After a second incubation period to permit the labeled antibody to complex with the antigen bound to the solid support or carrier through the unlabeled antibody, the solid support or carrier is washed a second time to remove the unreacted labeled antibody.

In another type of "sandwich" assay, which may also be useful with the antigens of the present invention, the so-called "simultaneous" and "reverse" assays are used. A simultaneous assay involves a single incubation step as the antibody bound to the solid support or carrier and labeled antibody are both added to the sample being tested at the same time. After the incubation is completed, the solid support or carrier is washed to remove the residue of fluid sample and uncomplexed labeled antibody. The presence of labeled antibody associated with the

solid support or carrier is then determined as it would be in a conventional "forward" sandwich assay.

In the "reverse" assay, stepwise addition first of a solution of labeled antibody to the fluid sample followed by the addition of unlabeled antibody bound to a solid support or carrier after a suitable incubation period is utilized. After a second incubation, the solid phase is washed in conventional fashion to free it of the residue of the sample being tested and the solution of unreacted labeled antibody. The determination of labeled antibody associated with a solid support or carrier is then determined as in the "simultaneous" and "forward" assays.

Immunoassays

The creation of immunoassays, such as RIA or ELISA, has been described in many articles, textbooks, and other publications. Reference is made to WO 97/03998, p. 48, line 4 to p. 52, line 27. Immunoassays of the invention may be if two general types: Firstly, immunoassays using immobilized TRAF2/NF-κB complex interacting protein, or an equivalent peptide, may be used in the quantification of caspase-8. Secondly, immunoassays using immobilized antibodies directed against an epitope of a TRAF2/NF-κB complex interacting protein may be used to quantify TRAF2/NF-κB complex interacting proteins.

Such assays may find use in diagnostics, as the level of caspase-8 and of other proteins involved in apoptotic pathways may need to be evaluated in a number of disorders or syndromes where involvement of such pathways is a possibility.

Nucleic acids

The clones obtained in the screening of the invention are expected to be partial clones. The obtention of complete

clones, if necessary, has been described further above and is exemplified further below. The DNA sequence of a complete clone and of the partial clone initially found in the screening of the invention may find a variety of uses.

For example, in order to manipulate the expression of a TRAF2/NF-κB complex interacting protein, it may be desirable to produce antisense RNA in a cell. To this end, the complete or partial cDNA coding for the TRAF2/NF-κB complex - interacting protein is inserted into an expression vector comprising a promoter, as noted further above. The 3′ end of the cDNA is thereby inserted adjacent to the 3′ end of the promoter, with the 5′ end of the cDNA being separated from the 3′ end of the promoter by said cDNA. Upon expression of the cDNA in a cell, an antisense RNA is therefore produced which is incapable of coding for the protein. The presence of antisense RNA in the cell reduces the expression of the cellular (genomic) copy of the TRAF2/NF-κB complex interacting protein gene.

For the production of antisense RNA, the complete cDNA may be used. Alternatively, a fragment thereof may be used, which is preferably between about 9 and 2,000 nucleotides in length, more preferably between 15 and 500 nucleotides, and most preferably between 30 and 150 nucleotides.

The fragment is preferably corresponding to a region within the 5′ half of the cDNA, more preferably the 5′ region comprising the 5′ untranslated region and/or the first exon region, and most preferably comprising the ATG translation start site. Alternatively, the fragment may correspond to DNA sequence of the 5′ untranslated region only.

A synthetic oligonucleotide may be used as antisense oligonucleotide. The oligonucleotide is preferably a DNA oligonucleotide. The length of the antisense oligonucleotide

is preferably between 9 and 150, more preferably between 12 and 60, and most preferably between 15 and 50 nucleotides. The region covered by the antisense oligonucleotide comprises preferably the 3′ untranslated region of the cDNA, more preferably it comprises the polyadenylation signal or the translation stop codon, or both.

The mechanism of action of antisense RNA and the current sate of the art of use of antisense tools is reviewed in Kumar et al. Microbiol Mol Biol Rev. 62, p. 1415-1434, 1998. The use of antisense oligonucleotides in inhibition of BMP receptor synthesis has been described by Yeh et al. J Bone Miner Res. 13,. p. 1870-9, 1998. The use of antisense oligonucleotides for inhibiting the synthesis of the voltage-dependent potassium channel gene Kv1.4 has been described by Meiri et al. PNAS 95, p. 15037-15042, 1998. The use of antisense oligonucleotides for inhibition of the synthesis of Bcl-x has been described by Kondo et al., Oncogene 17, p. 2585-91, 1998.

The therapeutic use of antisense drugs is discussed by Stix in Sci Am. 279, p. 46, 50, 1998, Flanagan, Cancer Metastasis Rev 17, p. 169-76, 1998, Guinot and Temsamani, Pathol Biol (Paris) 46, p. 347-54, 1998, and references therein.

Modifications of oligonucleotides that enhance desired properties are generally used when designing antisense oligonucleotides. For instance, phosphorothioate bonds are used instead of the phosphoester bonds naturally occurring in DNA, mainly because such phosphorothioate oligonucleotides are less prone to degradation by cellular enzymes. Peng et al. teach that undesired in vivo side effects of phosphorothioate oligonucleotides may be reduced when using a mixed phosphodiester-phosphorothioate backbone. Preferably, 2'-methoxyribonucleotide modifications in 60% of the

oligonucleotide is used. Such modified oligonucleotides are capable of eliciting an antisense effect comparable to the effect observed with phosphorothioate oligonucleotides. Peng et al. teach further that oligonucleotide analogs incapable of supporting ribonuclease H activity are inactive.

Therefore, the preferred antisense oligonucleotide of the invention has a mixed phosphodiester-phosphorothioate backbone. Most preferably, 2'-methoxyribonucleotide modifications in about 30% to 80%, most preferably about 60% of the oligonucleotide are used.

In the practice of the invention, antisense oligonucleotides or antisense RNA may be used. The length of the antisense RNA is preferably from about 9 to about 3,00 nucleotides, more preferably from about 20 to about 1,000 nucleotides, most preferably from about 50 to about 500 nucleotides.

In order to be effective, the antisense oligonucleotides of the invention must travel across cell membranes. In general, antisense oligonucleotides have the ability to cross cell membranes, apparently by uptake via specific receptors. As the antisense oligonucleotides are single-stranded molecules, they are to a degree hydrophobic, which enhances passive diffusion through membranes. Modifications may be introduced to an antisense oligonucleotide to improve its ability to cross membranes. For instance, the oligonucleotide molecule may be linked to a group comprising optionally partially unsaturated aliphatic hydrocarbon chain and one or more polar or charged groups such as carboxylic acid groups, ester groups, and alcohol groups. Alternatively, oligonucleotides may be linked to peptide structures, which are preferably membranotropic peptides. Such modified oligonucleotides penetrate membranes more easily, which is critical for their function and may therefore

significantly enhance their activity. Palmityl-linked oligonucleotides have been described by Gerster *et al.*, Anal. Biochem. 262, p. 177-84, 1998. Geraniol-linked oligonucleotides have been described by Shoji *et al.*, J. Drug Target 5, p. 261-73, 1998. Oligonucleotides linked to peptides, e.g., membranotropic peptides, and their preparation have been described by Soukchareun *et al.*, Bioconjug. Chem. 9, p. 466-75, 1998. Modifications of antisense molecules or other drugs that target the molecule to certain cells and enhance uptake of the oligonucleotide by said cells are described by Wang, J. Controlled Release 53, p. 39-48, 1998.

The antisense oligonucleotides of the invention are generally provided in the form of pharmaceutical compositions. Said compositions are for use by injection, topical administration, or oral uptake.

Preferred uses of the pharmaceutical compositions of the invention by injection are subcutaneous injection, intraperitoneal injection, and intramuscular injection.

The pharmaceutical composition of the invention generally comprises a buffering agent, an agent which adjusts the osmolarity thereof, and optionally, one or more carriers, excipients and/or additives as known in the art, e.g., for the purposes of adding flavors, colors, lubrication, or the like to the pharmaceutical composition.

Carriers may include starch and derivatives thereof, cellulose and derivatives thereof, e.g., microcrystalline cellulose, Xanthum gum, and the like. Lubricants may include hydrogenated castor oil and the like.

A preferred buffering agent is phosphate-buffered saline solution (PBS), which solution is also adjusted for osmolarity.

A preferred pharmaceutical formulation is one lacking a carrier. Such formulations are preferably used for administration by injection, including intravenous injection.

The preparation of pharmaceutical compositions is well known in the art and has been described in many articles and textbooks, see e.g., Remington's Pharmaceutical Sciences, Gennaro A. R. ed., Mack Publishing Company, Easton, Pennsylvania, 1990, and especially p 1521-1712 therein.

Additives may also be designed to enhance uptake of the antisense oligonucleotide across cell membranes. Such agents are generally agents that will enhance cellular uptake of double-stranded DNA molecules. For instance, certain lipid molecules have been developed for this purpose, including the transfection reagents DOTAP (Boehringer Mannheim), Lipofectin, Lipofectam, and Transfectam, which are available commercially. For a comparison of various of these reagents in enhancing antisense oligonucleotide uptake see e.g., Quattrone *et al.*, Biochemica 1, 25, 1995 and Capaccioli *et al.*, Biochem. Biophys. Res. Comm. 197, 818, 1993. The antisense oligonucleotide of the invention may also be enclosed within liposomes. The preparation and use of liposomes, e.g., using the above mentioned transfection reagents, is well known in the art. Other methods of obtaining liposomes include the use of Sendai virus or of other viruses. Examples of publications disclosing oligonucleotide transfer into cells using the liposome technique are e.g., Meyer *et al.*, J. Biol. Chem. 273, 15621-7, 1998, Kita and Saito, Int. J. Cancer 80, 553-8, 1999, Nakamura *et al.*, Gene Ther. 5, 1455-61, 1998, Abe et al., Antivir. Chem. Chemother. 9, 253-62, 1998, Soni *et al.*, Hepatology, 28, 1402-10, 1998, Bai *et al.*, Ann. Thorac. Surg. 66, 814-9, 1998, see also discussion in the same Journal p. 819-20, Bochot *et al.*, Pharm. Res. 15, 1364-9, 1998, Noguchi *et al.*, FEBS Lett. 433, 169-73, 1998, Yang *et al.*, Circ. Res.

83, 552-9, 1998, Kanamaru *et al.*, J. Drug Target. 5, 235-46, 1998, and references therein. The use of Lipofectin in liposome-mediated oligonucleotide uptake is described in Sugawa *et al.*, J. Neurooncol. 39, 237-44, 1998. The use of fusogenic cationic-lipid-reconstituted influenza-virus envelopes (cationic virosomes) is described in Waelti *et al.*, Int. J. Cancer, 77, 728-33, 1998.

The above-mentioned catioinic or nonionic lipid agents not only serve to enhance uptake of oligonucleotides into cells, but also improve the stability of oligonucleotides that have been taken up by the cell.

Ribozymes

Given the known mRNA sequence of a gene, ribozymes may be designed, which are RNA molecule that specifically bind and cleave said mRNA sequence (see e.g., Chen et al., Ann. NY Acad. Sci. 660, 271-3, 1992, Zhao and Pick, Nature 365, p. 448, 1993, Shore et al., Oncogene 8, 3183, 1993, Joseph and Burke, J. Biol. Chem. 268, 24515, 1993, Shimayama et al., Nucleic Acids Symp Ser 29, p. 177, 1993, Cantor et al., PNAS 90, p. 10932, 1993).

Accordingly, ribozyme-encoding RNA sequence may be designed that cleave the mRNA of a TRAF/NF-κB complex interacting protein of the invention. The point of cleavage is preferably located in the coding region or in the 5′ nontranslated region, more preferably, in the 5′ part of the coding region close to the AUG translation start codon.

A DNA encoding a ribozyme according to the invention may be introduced into cells by way of DNA uptake, uptake of modified DNA (see modifications for oligonucleotides and proteins that result in enhanced membrane permeability, as described hereinbelow), or viral vector-mediated gene transfer as detailed hereinbelow.

Introduction of TRAF2/NF-κB complex interacting proteins, peptides, and DNA into cells

The present invention provides TRAF2/NF-κB complex interacting proteins, peptides derived therefrom, antisense DNA molecules, and oligonucleotides. A therapeutic or research-associated use of these tools necessitates their introduction into cells of a living organism or into cultured cells. For this purpose, it is desired to improve membrane permeability of peptides, proteins and oligonucleotides. Ways to improve membrane permeability of oligonucleotides have been discussed above. The same principle, namely, derivatization with lipophilic structures, may also be used in creating peptides and proteins with enhanced membrane permeability. For instance, the sequence of a known membranotropic peptide as noted above may be added to the sequence of the peptide or protein. Further, the peptide or protein may be derivatized by partly lipophilic structures such as the above-noted hydrocarbon chains, which are substituted with at least one polar or charged group. For example, lauroyl derivatives of peptides have been described by Muranishi et al., Pharm. Research 8, 649, 1991. Further modifications of peptides and proteins comprise the oxidation of methionine residues to thereby create sulfoxide groups, as described by Zacharia et al., Eur. J. Pharmacol. 203, p. 353, 1991. Zacharia and coworkers also describe peptide or derivatives wherein the relatively hydrophobic peptide bond is replaced by its ketomethylene isoester (COCH2). These and other modifications known to the person of skill in the art of protein and peptide chemistry enhance membrane permeability.

Another way of enhancing membrane permeability is the use receptors, such as virus receptors, on cell surfaces in order to induce cellular uptake of the peptide or protein.

This mechanism is used frequently by viruses, which bind specifically to certain cell surface molecules. Upon binding, the cell takes the virus up into its interior. The cell surface molecule is called a virus receptor. For instance, the integrin molecules CAR and AdV have been described as virus receptors for Adenovirus, see Hemmi et al., Hum Gene Ther 9, p. 2363-73, 1998, and references therein. The CD4, GPR1, GPR15, and STRL33 molecules have been identified as receptors/coreceptors for HIV, see Edinger et al. Virology. 249, p. 367-78, 1998 and references therein.

Thus, conjugating peptides, proteins or oligonucleotides to molecules that are known to bind to cell surface receptors will enhance membrane permeability of said peptides, proteins or oligonucleotides. Examples for suitable groups for forming conjugates are sugars, vitamins, hormones, cytokines, transferrin, asialoglycoprotein, and the like molecules. Low et al., USP 5,108,921, describes the use of these molecules for the purpose of enhancing membrane permeability of peptides, proteins and oligonucleotides, and the preparation of said conjugates.

Low and coworkers further teach that molecules such as folate or biotin may be used to target the conjugate to a multitude of cells in an organism, because of the abundant and unspecific expression of the receptors for these molecules.

The above use of cell surface proteins for enhancing membrane permeability of a peptide, protein or oligonucleotide of the invention may also be used in targeting said peptide, protein or oligonucleotide of the invention to certain cell types or tissues. For instance, if it is desired to target cancer cells, it is preferable to use a cell surface protein that is expressed more abundantly on the surface of those cells. Examples are the folate receptor, the mucin antigens MUC1, MUC2, MUC3, MUC4, MUC5AC, MUC5B, and MUC7, the

glycoprotein antigens KSA, carcinoembryonic antigen, prostate-specific membrane antigen (PSMA), HER-2/neu, and human chorionic gonadotropin-beta. The above-noted Wang et al., 1998, teaches the use of folate to target cancer cells, and Zhang et al. Clin Cancer Res 4, p. 2669-76 1998, teaches the relative abundance of each of the other antigens noted above in various types of cancer and in normal cells.

The protein, peptide or oligonucleotide of the invention may therefore, using the above-described conjugation techniques, be targeted to certain cell type as desired. For instance, if it is desired to enhance NF-κB induction in cells of the lymphocytic lineage, a NF-κB complex positive modulating protein or peptide of the invention may be targeted at such cells, for instance, by using the MHC class II molecules that are expressed on these cells, or by using the IL-2 receptor marker which preferably appears on the surface of activated cells. The skilled person will recognize the possibilities of using a cell surface marker selected from a multitude of known markers of lymphoid and other cells, depending ion the cell type to be targeted, and of these, further selecting those that are expressed constitutively or inducibly. For instance, if it is desired to reduce NF-κB induction in the context of an autoimmune disease, it may be advantageous, according to the invention, to use a cell surface marker that is induced when lymphoid cells are activated. Specific markers that are activated in the context of the specific autoimmune disease to be treated are preferred. For instance, in rheumatoid arthritis, the expression of cell surface markers ICAM-1, PECAM-1 and E selectin is up-regulated in rheumatic nodules and/or nearby blood vessels, while the T cell marker , CD30, appears to be up-regulated in T cells involved in the disease process (see e.g., Elewaut et al., Ann Rheum Dis 57, 480-6, 1998)

This may be achieved by coupling an antibody, or the antigen-binding site thereof, directed against the constant region of said MHC class II molecule or against another desired cell surface marker as mentioned above, to the protein or peptide of the invention. Further, numerous cell surface receptors for various cytokines and other cell communication molecules have been described, and many of these molecules are expressed with in more or less tissue- or cell-type restricted fashion. Thus, when it is desired to target a subgroup of T cells, the CD4 T cell surface molecule may be used for producing the conjugate of the invention. CD4-binding molecules are provided by the HIV virus, whose surface antigen gp42 is capable of specifically binding to the CD4 molecule. An TRAF2/NF-κB complex downmodulating protein or peptide of the invention may be advantageously targeted to T cells in the treatment of patient who suffer from autoimmune reactions based upon T cells, such as lupus erythematodes patients. An example for such a suitable protein according to the invention would be the protein encoded by clone 10, or derivatives, isoforms or fragments thereof with equal biologically activity.

Virus-mediated cellular targeting

The proteins, peptides and antisense sequences of the invention may be introduced into cells by the use of a viral vector. The use of vaccinia vector for this purpose is detailed in the above-noted chapter 16 of Current Protocols in Molecular Biology. The use of adenovirus vectors has been described e.g. by Teoh et al., Blood 92, p. 4591-4601, 1998, Narumi et al., Am J Respir Cell Mol Biol 19, p. 936-941, 1998, Pederson et al, J Gastrointest Surg 2, p. 283-91, 1998, Guang-Lin et al., Transplant Proc 30, p. 2923-4, 1998, and references therein, Nishida et al., Spine 23, p. 2437-42,

1998, Schwarzenberger et al., J Immunol 161, p. 6383-9, 1998, and Cao et al., J Immunol 161, p. 6238-44, 1998. Retroviral transfer of antisense sequences has been described by Daniel et al. J Biomed Sci. 5, p. 383-94, 1998. The use of SV-40 derived viral vectors and SV-40 based packaging systems has been described by Fang et al., Anal. Biochem. 254, 139-43, 1997. The use of papovaviruses which specifically target B-lymphocytes, has been described by Langner et al., Adv. Exp. Med. Biol. 451, 415-22, 1998.

When using viruses as vectors, the viral surface proteins are generally used to target the virus. As many viruses, such as the above adenovirus, are rather unspecific in their cellular tropism, it may be desirable to impart further specificity by using a cell-type or tissue-specific promoter. Griscelli et al., Hum Gene Ther. 9, p. 1919-28, 1998 teach the use of the ventricle-specific cardiac myosin light chain 2 promoter for heart-specific targeting of a gene whose transfer is mediated by adenovirus.

Alternatively, the viral vector may be engineered to express an additional protein on its surface, or the surface protein of the viral vector may be changed to incorporate a desired peptide sequence. The viral vector may thus be engineered to express one or more additional epitopes which may be used to target said viral vector. For instance, cytokine epitopes, MHC class II-binding peptides, or epitopes derived from homing molecules may be used to target the viral vector in accordance with the teaching of the invention. The above Langer et al., 1998, teach the use of heterologous binding motifs to target B-lymphotrophic papoaviruses.

The pharmaceutical compositions of the invention are prepared generally as known in the art. Thus, pharmaceutical compositions comprising nucleic acid, e.g., ribozymes, antisense RNA or antisense oligonucleotides, are prepared as

detailed above for pharmaceutical compositions comprising oligonucleotides and/or antisense RNA. The above consideration apply generally also to other pharmaceutical compositions. For instance, the pharmaceutical composition of the invention may comprise naked DNA, e.g. DNA encoding the NAP protein or isoforms, fragments or derivatives thereof and pharmaceutically acceptable carrier as known in the art. A variety of ways to enhance uptake of naked DNA is known in the art. For instance, cationic liposomes (Yotsuyanagi and Hazemoto, Nippon Rinsho 56, 705-12, 1998), dicationic amphiphiles (Weissig et al., Pharm. Res. 15, 334-7, 1998), fusogenic liposomes , (Mizuguchi et al., Biochem. Biophys. Res. Commun. 218, 402-7, 1996), mixtures of stearyl-poly(L-lysine) and low density lipoprotein (LDL), (terplex, Kim et al., J. Controlled. Release 53, 175-82, 1998), and even whole bacteria of an attenuated mutant strain of Salmonella Typhimurium (Paglia et al., Blood 92, 3172-6, 1998) have been used in the preparation of pharmaceutical compositions containing DNA.

Administration of virus particles has been described in prior art publications, see e.g., United States Patent 5,882,877, wherein Adenovirus based vectors and administration of the DNA thereof is described. The viral DNA was purified on a CsCl gradient and then dialyzed against tris-buffered saline to remove the CsCl. In these preparations, viral titers (pfu/ml) of 14^{14} to 10^{10} are preferably used. Administration of virus particles as a solution in buffered saline, to be preferably administered by subcutaneous injection, is known from United States Patent 5,846,546. Croyle and coworkers describe a process for the preparation of a pharmaceutical composition of recombinant adenoviral vectors for oral gene delivery, using CsCl gradients and lyophilization in a sucrose-containing buffer (Pharm. Dev. Technol. 3, 365-72, 1998).

Where the pharmaceutical composition of the invention comprises a peptide or protein according to the invention, the composition will generally contain salts, preferably in physiological concentration, such as PBS (phosphate-buffered saline), or sodium chloride (0.9% w/v), and a buffering agent, such as phosphate buffer in water or in the well-known PBS buffer. In the following section, the term "peptide is meant to include all proteins or peptides according to the invention. The preparation of pharmaceutical compositions is well known in the art, see e.g., US Patents 5,736,519, 5,733,877, 5,554,378, 5,439,688, 5,418,219, 5,354,900, 5,298,246, 5,164,372, 4,900,549, 4,755,383, 4,639,435, 4,457,917, and 4,064,236. The peptide of the present invention, or a pharmacologically acceptable salt thereof is preferably mixed with an excipient, carrier, diluent, and optionally, a preservative or the like pharmacologically acceptable vehicles as known in the art, see e.g., the above US patents. Examples of excipients include, glucose, mannitol, inositol, sucrose, lactose, fructose, starch, corn starch, microcrystalline cellulose, hydroxypropylcellulose, hydroxypropyl- methylcellulose, polyvinylpyrrolidone and the like. Optionally, a thickener may be added, such as a natural gum, a cellulose derivative, an acrylic or vinyl polymer, or the like.

The pharmaceutical composition is provided in solid, liquid or semi-solid form. A solid preparation may be prepared by blending the above components to provide a powdery composition. Alternatively, the pharmaceutical composition is provided as lyophilized preparation. The liquid preparation is provided preferably as aqueous solution, aqueous suspension, oil suspension or microcapsule composition. A semi-solid composition is provided preferably as hydrous or oily gel or

ointment. About 0.001 to 60 w/v %, preferably about 0.05 to 25 w/v % of peptide is provided in the composition.

A solid composition may be prepared by mixing an excipient with a solution of the peptide of the invention, gradually adding a small quantity of water, and kneading the mixture. After drying, preferably *in vacuo*, the mixture is pulverized. A liquid composition may be prepared by dissolving, suspending or emulsifying the peptide of the invention in water, a buffer solution or the like. An oil suspension may be prepared by suspending or emulsifying the peptide of the invention or protein in an oleaginous base, such as sesame oil, olive oil, corn oil, soybean oil, cottonseed oil, peanut oil, lanolin, petroleum jelly, paraffin, Isopar, silicone oil, fatty acids of 6 to 30 carbon atoms or the corresponding glycerol or alcohol esters. Buffers inlcude Sorensen buffer (Ergeb. Physiol., 12, 393 1912), Clark-Lubs buffer (J. Bact., 2, (1), 109 and 191, 1917), Macllvaine buffer (J. Biol. Chem., 49, 183, 1921), Michaelis buffer (Die Wasserstoffinonenkonzentration, p. 186, 1914), and Kolthoff buffer (Biochem. Z., 179, 410, 1926).

A composition may be prepared as a hydrous gel, e.g. for transnasal administration. A hydrous gel base is dissolved or dispersed in aqueous solution containing a buffer, and the peptide of the invention, and the solution warmed or cooled to give a stable gel.

Preferably, the peptide of the invention is administered through intravenous, intramuscular or subcutaneous administration. Oral administration is expected to be less effective, because the peptide may be digested before being taken up. Of course, this consideration may apply less to a peptide of the invention which is modified, e.g., by being cyclic peptide, by containing non-naturally occurring amino acids, such as D-amino acids, or other modification

which enhance the resistance of the peptide to biodegradation. Decomposition in the digestive tract may be lessened by use of certain compositions, for instance, by confining the peptide of the invention in microcapsules such as liposomes. The pharmaceutical composition of the invention may also be administered to other mucous membranes. The pharmaceutical composition is then provided in the form of a suppository, nasal spray or sublingual tablet. The dosage of the peptide of the invention may depend upon the condition to be treated, the patient's age, bodyweight, and the route of administration, and will be determined by the attending physician.

The uptake of a peptide of the invention may be facilitated by a number of methods. For instance, a non-toxic derivative of the cholera toxin B subunit, or of the structurally related subunit B of the heal-labile enterotoxin of enterotoxic Eschericia coli may be added to the composition, see USP 5,554,378.

In another embodiment, the peptide of the invention is provided in a pharmaceutical composition comprising a biodegradable polymer selected from poly-1,4-butylene succinate, poly-2,3-butylene succinate, poly-1,4-butylene fumarate and poly-2,3-butylene succinate, incorporating the peptide of the invention as the pamoate, tannate, stearate or palmitate thereof. Such compositions are described e.g., in USP 5,439,688.

In another embodiment, a composition of the invention is a fat emulsion. The fat emulsion may be prepared by adding to a fat or oil about 0.1-2.4 w/w of emulsifier such as a phospholipid, an emulsifying aid, a stabilizer, mixing mechanically, aided by heating and/or removing solvents, adding water and isotonic agent, and optionally, adjusting adding the pH agent, isotonic agent. The mixture is then homogenized. Preferably, such fat emulsions contain an

electric charge adjusting agent, such as acidic phospholipids, fatty acids, bilic acids, and salts therof. Acidic phospholipids include phosphatidylserine, phosphatidylglycerol, phosphatidylinositol, and phosphatidic acid. Bilic acids include deoxycholic acid, and taurocholic acid. The preparation of such pharmaceutical compositions is described in US 5,733,877.

Examples

Materials and Methods

1. B-cell cDNA library

An oligo dT primed library prepared from human B cells was used (Durfee, T. *et al.* (1993) Genes Dev. 7: 555-569). The cDNAs of this library were inserted into the XhoI site of the pACT based vector pSE1107 in fusion with the GAL4 activation domain.

2. Yeast strain

The HF7c strain was used as the host strain for the purposes of transformation and screening with the two-hybrid assay. This strain carries the auxotrophic markers trp1 and leu2, and therefore cannot grow in minimal synthetic medium that lacks tryptophan and leucine, unless they also bear a plasmid carrying the wild-type versions of the genes TRP1 and LEU2. The HF7c strain also carries deletion mutations in its GAL4 and GAL80 genes (gal4-542 and gal80-538, respectively). This strain also carries the lacZ reporter gene in its genotype, fused to three copies of the GAL4 17-mer consensus sequence and the TATA portion of the CYC1 promoter. The GA4 17-mers are responsive to the GAL4 transcriptional activator.

In addition, this yeast strain also carries the HIS3 reporter fused to the UAS and the TATA portion of the GAL1 promoter.

3. Two-hybrid screen of B-library

The two hybrid screen is a used in order to identify factors that are associated with a particular molecule that serves as a "bait". In the present invention NEMO that was cloned into the vector pGBT9, served as the bait. NEMO was co-expressed together with the screened B-cell cDNA library in the yeast strain HF7c. The PCR-cloned NEMO was a recombinant fusion with the CAL4 DNA-binding domain and the screened cDNA library was fused to the GAL4 activation domain in the pSE1107 vector. The reporter gene in HF7c was HIS3 fused to the upstream activating sequence (UAS) of the GAL1 promoter which is responsive to GAL4 transcriptional activator. Transformants that contained both pGBT9 and pSE1107 plasmids were selected for growth on plates without tryptophan and leucine. In a second step positive clones which expressed two hybrid proteins that interact with each other, and therefore activated GAL1-HIS3, were picked up from plates devoided of tryptophan, leucine and histidine and contained 50 mM 3-aminotriazol (3AT).

4. β-galactosidase assay

Positive clones picked up in the two hybrid screen were subjected to lacZ color development test in SFY526 yeast cells, following Clontech Laboratories' manual (for details see above mentioned publications and patent applications). In brief, transformants were allowed to grow at 30^{0}C for 2-4 days until reaching about 2 mm in diameter, then were transferred onto Whatman filters. The filters went through a freeze/thaw treatment in order to permeabilize the cells, then soaked in a buffer (16.1 mg/ml $Na_2HPO_4 \cdot 7H_2O$; 5.5 mg/ml $NaH_2PO_4 \cdot H_2O$; 0.75

mg/ml KCl; 0.75 mg/ml $MgSO_4 \cdot 7H_2O$, pH=7) containing 0.33 mg/ml X-gal and 0.35 mM β-mercaptoethanol. Colonies were monitored for development of blue color which is an indication for induction of β-galactosidase.

5. Expression of cloned cDNAs

Two kinds of expression vectors were constructed:

a) A pUHD10-3 based vectors containing the open reading frame (ORF) of the relevant clones, fused to the Hemeaglutinine (HA) epitope.

b) A pUHD10-3 based vector into which FLAG octapeptide sequence was introduced just in front of the insert.

6. Immunoprecipitation and Western blot analysis

Human embryonic kidney 293-EBNA cells (Invitrogen) were transfected in aliquots of $4x10^6$ ($2\ x10^6$ per 10-cm dish, 20 μg of DNA per dish) by the calcium phosphate method. Twenty-six hours after transfection the cells were lysed in buffer containing 50 mM HEPES pH 7.5, 250mM NaCl, 0.2% NP-40, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin and 20 μg/ml leupeptin (lysis buffer). Immunoprecipitation was performed by co-incubation (2h, 4^0C) of 1-ml aliquots of lysate ($2\ x10^6$ cells per aliquot) with an antibody of interest and with protein G-agarose beads (30 μl/aliquot). Immunoprecipitates were washed three times with lysis buffer and once with PBS, fractionated by 10% SDS-PAGE and transferred onto a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). Western blot analysis was performed with a relevant antisera, applied at a dilution of 1:1000, and the ECL kit (as specified by the manufacturer: Amersham, Buckinghamshire, England).

Example 1

Cloning

A cDNA library prepared from B-cells was screened for proteins that associate with NEMO, using the two hybrid technique as described in Materials and Methods. Only in transformants that expressed both NEMO and a protein capable of interacting with it, the GAL4 DNA-binding domain and the transcriptional activation domain were brought together. The result was the activation and expression of the reporter gene, in this case HIS3 fused to the UAS and the TATA portion of the GAL1 promoter.

The screen yielded approximately 2000 clones which were able to grow on Trp-, Leu-, His- 3AT plates. DNA prepared from 165 randomly selected positive clones served for transient co-transfection of SFY526 yeast srain together with TRAF2 cloned into pGBT9 vector. Assay for β-galactosidase activity was performed on the transformed SFY526 yeast colonies as described in Materials and Methods. The blue color that developed was an indication for yeast colonies that contain cDNA encoding a protein or polypeptide that binds to NEMO.

The results of a two hybrid specificity test carried out on one of the clones, clone 10, together with various signaling proteins, are summarized in Table II, below. Faster binding kinetics is indicative of a stronger interaction. The proteins that bind specifically to clone 10 (RAP-2 and TRAF2) are shown in bold type in Table II.

Table II

Gal-BD Construct	Kinetics of Int-n with Gal-AD-clone 10

Bcl-2	>12 hrs.
Cyclin D	>12 hrs.
ICE	>12 hrs.
Lamin	>12 hrs.
MACH	>12 hrs.
MORT-1	>12 hrs.
NIK	>12 hrs.
RAP-2	**20 min.**
RIP	>12 hrs.
TRADD	>12 hrs.
TRAF-2	**20 min.**

Applying several PCR steps to cDNA clone 10, the full length cDNA was cloned from cDNA libraries obtained from RNA of human tissues. This cDNA clone was designated clone compl. 10 (i.e. complete clone 10).

Example 2

Sequencing new clones

Cloned cDNAs (obtained as described above in Example 1) were purified, amplified in E. coli and the DNA obtained therefrom was subject to sequence analysis using an ABI automatic sequencer. The cDNA sequences of clone 10 and of clone compl. 10 are shown in figures 1 and 2 respectively. The amino acid sequence of the NAP polypeptide, as deduced from the cDNA sequence of clone compl. 10 is shown in figure 3.

Example 3

Expression of cloned cDNAs and interaction between the expressed proteins

HeLa-Bujard cells were transfected with NEMO tagged with FLAG in pUHD10-3 based expression vector and constructs containing ORF of the selected clones fused to HA epitope, above. Cells were then grown for 24 hrs. in Dulbecco's Modified Eagle's Medium (DMEM) plus 10% calf serum with added 35S-Methionine and 35S-Cysteine. At the end of that incubation time cells were lysed in radioimmune precipitation buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonident P-40, 1% deoxycholate, 0.1% SDS, and 1 mM EDTA; 1 ml/ 5x105 cells), and the lysate was precleared by incubation with irrelevant rabbit antiserum and Protein G-Sepharose beads (Pharmacia, Sweden). Immunoprecipitation was performed by 1 hour incubation at 4oC of aliquots of the lysate with anti-FLAG (purchased from Eastman Kodak Co.) or anti-HA (clone 12CA5 (Field, J. et al. (1988)) monoclonal antibodies. The expressed proteins were analysed on SDS-PAGE gel followed by autoradiography.

The results of such experiments demonstrated that the partial cDNA clone 10 encoded a protein of molecular weight around 65kDa.

While specific embodiments of the invention have been described for the purpose of illustration, it will be understood that the invention may be carried out in practice by skilled persons with many modifications, variations and adaptations, without departing from its spirit or exceeding the scope of the claims.

CLAIMS

1. An isolated nucleotide sequence encoding a protein NAP, its isoforms, fragments and derivatives, capable of binding to tumor necrosis factor receptor-associated factor 2 (TRAF2), and to at least one component of the signalosome.

2. An isolated nucleotide sequence according to claim 1, wherein said at least one component of the signalosome is the NF-κB essential modifier (NEMO).

3. A nucleotide sequence selected from the group consisting of:

One) nucleotide sequences capable of hybridization to a sequence of claim 1 or 2 under moderately stringent conditions;

Two) a nucleotide sequence capable of priming a DNA or RNA polymerase when annealed to a sequence of (a); and

c) nucleotide sequences analogous to sequences of claims 1 or 2 within the degeneracy of the genetic code and which encode a protein capable of binding to a TRAF2, and to at least one component of the signalosome, or a functional isoform, fragment or derivative thereof.

4. A nucleotide sequence according to claim 1 or claim 2, which is a nucleotide sequence of the herein designated clone 10 comprising the nucleotide sequence depicted in Fig 1, or a nucleotide sequence of the herein designated clone compl. 10 comprising the nucleotide sequence depicted in Fig. 2.

5. A nucleotide sequence according to claim 1 or claim 2, comprising the DNA sequence encoding the protein NAP substa ntially as shown in Fig. 3, or a functional fragment thereof.

6. A nucleotide sequence encoding the protein NAP, isoforms, fragments or analogs thereof, said NAP, isoforms, fragments or analogs thereof being capable of binding independently both to TRAF2 and to NEMO, and which is capable of modulating/mediating the activity of NF-κB.

7. A nucleotide sequence according to claim 6, selected from the group consisting of:

a) a nucleotide sequence derived from the coding region of a native NAP protein;

b) nucleotide sequences capable of hybridization to a sequence of (a) under moderately stringent conditions and which encode a biologically active NAP protein; and

c) nucleotide sequences analogous to sequences of (a) or (b) within the degeneracy of the genetic code and which encode a biologically active NAP protein.

8. A vector comprising a DNA sequence according to any one of claims 1 and 2 and 4 to 7.

9. A vector according to claim 8 capable of being expressed in a prokaryotic host cell or capable of being expressed in a eukaryotic host cell.

10. Eukaryotic or prokaryotic host cells transformed with a vector according to claim 8 or 9.

11. A protein capable of binding to a component of the signalosome and to TRAF2, and to a functional isoforms, fragments, analogs and derivatives thereof.

12. A protein capable of binding to TRAF2, and to a component of the signalosome, and isoforms, fragments, analogs and derivatives thereof, encoded by a DNA sequence of any one of claims 1 and 2 and 4 to 7.

13. A protein according to claim 12 being the protein encoded by clone 10, or the protein encoded by clone compl. 10.

14. A protein according to claim 13 being the protein encoded by clone compl. 10.

15. A protein according to claim 12 being the NAP protein, functional isoforms, analogs, fragments and derivatives thereof, encoded by the DNA sequence according to any one of claims 1 and 2 and 4 to 7.

16. The NAP protein, functional isoforms, analogs, fragments and derivatives thereof according to claim 15, having at least part of the amino acid sequence depicted in Fig. 3.

17. A method for producing a protein, isoform, fragment, analog or derivative thereof according to any one of claims 11-15, which comprises growing a transformed host cell according to claim 10 under conditions suitable for the expression of said protein, isoform, fragment, analog or derivative thereof, isolating said expressed protein, isoform, fragment, analog or derivative.

18. Antibodies or active fragments or derivatives thereof, specific for the TRAF2/signalosome-binding protein, isoform, analog, fragment or derivative thereof according to

claims 11 to 14; or specific for the NAP protein, isoform, analog, fragment or derivative thereof according to claim 15 or 16.

19. A method for the modulation or mediation in cells of the activity of NF-κB or any other intracellular signaling activity modulated or mediated by TRAF2 or by other molecules to which a protein, isoform, analog, fragment or derivative thereof according to any one of claims 11 to 15 binds, said method comprising treating said cells by introducing thereinto one or more of said protein, isoform, analog, fragment or derivative thereof in a form suitable for intracellular introduction thereof, or introducing into said cells a DNA sequence encoding said one or more protein, isoform, analog, fragment or derivative thereof in the form of a suitable vector carrying said sequence, said vector being capable of effecting the insertion of said sequence into said cells in a way that said sequence is expressed in said cells.

20. A method according to claim 19 wherein said treating of said cells is by transfection of said cells with a recombinant animal virus vector comprising the steps of:

(a) constructing a recombinant animal virus vector carrying a sequence encoding a viral surface protein (ligand) that is capable of binding to a specific cell surface receptor on the surface of said cells to be treated and a second sequence encoding a protein selected from the said protein, isoforms, analogs, fragments and derivatives according to any one of claims 13 to18, that when expressed in said cells is capable of modulating/mediating the activity of NF-κB or any other intracellular signaling activity modulated/mediated by TRAF2; and

(b) infecting said cells with said vector of (a).

21. A method for modulating/mediating TRAF2/NF-κB comprising treating said cells with antibodies or active fragments or derivatives thereof according to claim 18, said treatment being by application of a suitable composition containing said antibodies, active fragments or derivatives thereof to said cells, wherein when the cellular TRAF2/NF-κB complex-binding protein or fragment thereof appears as extracellular surface protein, said composition is formulated for extracellular application, and when said TRAF2/NF-κB complex-binding proteins are intracellular said composition is formulated for intracellular application.

22. A method for modulating/mediating TRAF2/NF-κB comprising treating said cells with an antisense oligonucleotide targeted at at least part of the RNA of the TRAF2/NF-κB complex-interacting protein according to any one of claims 1 and 2 and 4 to 7, said antisense oligonucleotide sequence being capable of reducing the expression of the TRAF2/signalosome-interacting protein.

23. A method according to claim 22 wherein said antisense oligonucleotide sequence is introduced into said cells by means of an animal virus comprising the same.

24. A method for modulating/mediating TRAF2/NF-κB comprising introducing into a cell a ribozyme sequence capable of interacting with a cellular mRNA sequence encoding a TRAF/NF-κB complex-binding protein according to any one of claims 11 to16, or a vector comprising said ribozyme sequence, whereby expression of said protein is inhibited.

25. A method for isolating and identifying proteins, according to any one of claims 11 to16, capable of binding directly to TRAF2/NF-κB complex, comprising applying the yeast two-hybrid procedure in which a sequence encoding said TRAF2 is carried by one hybrid vector and sequence from a cDNA or genomic DNA library is carried by the second hybrid vector, the vectors then being used to transform yeast host cells and the positive transformed cells being isolated, followed by a second two-hybrid test of TRAF2-binding clones for binding to a component of the NF-κB complex, further followed by selection of those clones that bind to TRAF2 and to said component of the signalosome, and still further, followed by extraction of the said second hybrid vector to obtain a sequence encoding a protein which binds to said TRAF2 and to said component of the signalosome.

26. A method according to any one of claims 19 to25 wherein said protein is NAP or at least one of the NAP isoforms, analogs, fragments and derivatives thereof.

27. A pharmaceutical composition for the modulation/mediating of TRAF2 comprising, as active ingredient at least one TRAF2/signalosome-interacting protein, according to any one of claims 11 to 16, its biologically active fragments, analogs, derivatives or mixtures thereof.

28. A pharmaceutical composition for modulating/mediating TRAF2/NF-κB comprising, as active ingredient, a recombinant animal virus vector encoding a protein capable of binding a cell surface receptor and encoding at least one TRAF2/signalosome-interacting protein, isoform, active fragments or analogs, according to any one of claims 11 to16.

29. A pharmaceutical composition for modulating/mediating TRAF2/NF-κB comprising as active ingredient, an antisense oligonucleotide of the TRAF2/signalosome interacting protein mRNA sequence according to any one of claims 1 and 2 and 4 to7.

30. A pharmaceutical composition for the prevention or treatment of a pathological condition associated with NF-κB induction or by other molecules to which a protein according to any one of claims 11-16 binds, said composition comprising an effective amount of a protein encoded by a DNA molecule of any one of claims 1 and2 and 4 to 7, or a molecule capable of disrupting the interaction of said protein with TRAF2/signalosome or any other molecule to which said protein binds.

31. A pharmaceutical composition for the prevention or treatment of a pathological condition associated with NF-κB induction or with other molecules to which a protein according to any one of claims 11 to16 binds, said composition comprising an effective amount of a NAP protein, isoform, fragment, analog or derivative thereof, or a DNA molecule coding therefor, or a molecule capable of disrupting the interaction of said NAP protein, isoform, fragment, analog or derivative thereof with TRAF2 and/or with the signalosome or any other molecule to which said NAP protein, isoform, fragment, analog or derivative binds.

32. A pharmaceutical composition for the prevention or treatment of a pathological condition associated with NF-κB induction, said composition comprising an effective amount of a protein of claim 11 or 12 or a DNA molecule coding therefor,

or a molecule capable of disrupting the interaction of said protein of claim 11 or 12 with TRAF2 or with the signalosome component to which it binds, or with any other molecule to which said protein binds.

33. A pharmaceutical composition for the prevention or treatment of a pathological condition associated with NF-κB induction or with any other activity mediated by interaction with an NAP protein, an isoform, fragment, analog or derivative thereof, said composition comprising an effective amount of an NAP protein, isoform, fragment, analog or derivative thereof, or a DNA molecule coding therefor, or a molecule capable of disrupting the interaction of said NAP protein, isoform, fragment, analog or derivative thereof with TRAF2 or a signalosome component or any other molecule to which said NAP protein, isoform, fragment, analog or derivative binds.

34. A method for the prevention or treatment of a pathological condition associated with NF-κB induction, said method comprising administering to a patient in need an effective amount of a protein or isoform, fragment, analog and derivative thereof or a mixture of any thereof according to any one of claims 11 to 16, or a DNA molecule coding therefor, or a molecule capable of disrupting the interaction of said protein or isoform, fragment, analog and derivative thereof or a mixture of any thereof according to any one of claims 11 to 16 with TRAF2 or with a component of the signalosome or any other molecule to which said protein or isoform, fragment, analog and derivative thereof or a mixture of any thereof according to any one of claims 11-16 binds.

35. A method according to claim 34 wherein said protein is encoded by clone 10, or by clone compl. 10.

36. A method according to claim 34, wherein said protein is NAP.

37. A method for screening of a ligand capable of binding to a protein according to any one of claims 11 to 16 comprising contacting an affinity chromatography matrix to which said protein is attached with a cell extract whereby the ligand is bound to said matrix, and eluting, isolating and analyzing said ligand.

38. A method of screening for a DNA sequence coding for a ligand capable of binding to a protein according to any one of claims 11 to 16 comprising applying the yeast two-hybrid procedure in which a sequence encoding said protein is carried by one hybrid vector and sequences from a cDNA or genomic DNA library are carried by the second hybrid vector, transforming yeast host cells with said vectors, isolating the positively transformed cells, and extracting said second hybrid vector to obtain a sequence encoding said ligand.

39. A method for identifying and producing a ligand capable of modulating the cellular activity modulated/mediated by TRAF2 and/or NEMO comprising :

a) screening for a ligand capable of binding to TRAF2;

b) identifying and characterizing a ligand, other than TRAF2 or portions of a receptor of the TNF/NGF receptor family, found by said screening step to be capable of said binding;

c) testing a ligand identified in step (b) for binding to at least a portion of NEMO having the amino acid residues 218 to 416 of NEMO, and selecting a ligand that does bind to said NEMO; and

d) producing said ligand in substantially isolated and purified form.

40. A method for identifying and producing a ligand capable of modulating the cellular activity modulated or mediated by a protein according to any one of claims 11-16 comprising :

a) screening for a ligand capable of binding to a polypeptide comprising at least a portion of the NAP sequence depicted in Fig. 3;

b) identifying and characterizing a ligand, other than TRAF2 found by said screening step to be capable of said binding; and

c) testing a ligand identified in step (b) for binding to at least a portion of NEMO having the amino acid residues 218 to 416 of NEMO, and selecting a ligand that does bind to said NEMO; and

d) producing said ligand in substantially isolated and purified form.

41. A method for identifying and producing a ligand capable of modulating the cellular activity modulated/mediated by NAPcomprising:

a) screening for a ligand capable of binding to at least a portion of the NAP sequence depicted in Fig. 3;

b) identifying and characterizing a ligand, other than TRAF2, found by said screening step to be capable of said binding; and

c) testing a ligand identified in step (b) for binding to at least a portion of NEMO having the amino acid residues 218 to 416 of NEMO, and selecting a ligand that does bind to said NEMO; and

d) producing said ligand in substantially isolated and purified form.

42. A method for identifying and producing a molecule capable of directly or indirectly modulating the cellular activity modulated/mediated by NAP, comprising :

a) screening for a molecule capable of modulating activities modulated/mediated by NAP;

b) identifying and characterizing said molecule; and

c) producing said molecule in substantially isolated and purified form.

43. An isolated nucleotide sequence comprising the clone-10, or clone compl. 10, substantially as described and illustrated.

44. An expression vector comprising a DNA sequence of claim 43, substantially as described and illustrated.

45. A method of identifying TRAF2/NEMO binding proteins, substantially as described and illustrated.

46. A method of modulating the activity of NF-κB, substantially as described and illustrated.

For the Applicants

Henry Einav

NAP PROTEIN, ITS PREPARATION

AND USE

חלבון NAP, הכנתו והשימוש בו

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